Influence of ApoE on LRP1 Function and Amyloid Transport Across the Blood-Brain Barrier

Thesis

Influence of ApoE on LRP1 Function and Amyloid Transport Across the Blood-Brain Barrier

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A thesis submitted for the degree of Doctor of Philosophy in the discipline of Neuroscience

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Declaration:

I hereby declare that the work presented in this thesis is my own, except for where stated. This work has not been submitted for any other degree of professional qualification.

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Abstract

Alzheimer's disease is a progressive incurable neurological disorder and is the leading cause of dementia in the elderly. Common symptoms include failure of memory, language and mood disturbances confusion and depression. The clinical duration of Alzheimer's disease is approximately 8-10 years post-diagnosis and patients require increasing assistance throughout its progression. The currently available treatments are palliative, focussing on improving cognition and only result in mild improvements in small subsets of patients for a relatively short period of time. It is therefore clear that new approaches are needed if a therapy is to be developed that has the potential to be disease modifying and slows the progression of Alzheimer's disease.

One of the main pathological hallmarks of Alzheimer's disease is the accumulation of beta-amyloid (Aβ) peptides in the brain. In particular the soluble Aβ species has been linked to neurotoxicity, reductions in long term potentiation, and can induce cognitive deficits in animal models of AD after intracerebral injection. In addition, increased accumulation of Aβ in the brain is also associated with the APOE4 allele, which is the strongest genetic factor for developing Alzheimer's disease. APOE exists as three alleles with the two copies of the APOE4 allele conferring a 15-fold increase in the risk of developing AD when compared to APOE3 homozygous individuals.

Recently it has been demonstrated that this accumulation occurs as a result of defective clearance mechanisms. One of the main routes of clearance of Aβ is through the blood-brain barrier (BBB) where specialised transporters facilitate its clearance. The Low Density Lipoprotein receptor-related protein (LRP1) plays a prominent role in the BBB clearance of Aβ and its surface expression is at least in part regulated by ectodomain cleavage by a variety of 'sheddases'. In particular, this thesis focuses on the previously identified LRP1 sheddases ADAM10 and MMP9. The shedding of LRP1 produces a soluble LRP1 fragment, which is unable to endocytose and subsequently transcytose bound ligands.

In this thesis, I examined the role of apoE in the regulation of LRP1 and the transcytosis and clearance of Aβ through the BBB. I demonstrate that the shedding of LRP1 is modulated by the different apoE isoforms and that inhibition of LRP1 sheddases increases the clearance of Aβ through the BBB. MMP9 is the primary candidate as the main driver of this mechanism due to its apoE isoform dependent activity and increased expression in AD. In summary, I identified a novel target for treatment strategies that facilitates the clearance of Aβ though the BBB. This approach may be particularly effect in individuals with the APOE4 allele, who are in desperate need of viable therapeutics to combat AD.

Contents

Tables and Figures:

List of Abbreviations:

AD	Alzheimer's disease
ABCA1	ATP binding cassette 1
ADAM	A disintegrin and metalloproteinase
AICD	Amyloid Precursor Protein Intracellular Domain
ANOVA	Analysis of Variance
APOE	Apolipoprotein E gene
apoE	Apolipoprotein E protein
APOE-TR	APOE Targeted Replacement
APP	Amyloid Precursor Protein
ARIA	Amyloid-Related Imaging Abnormalities
ATP	Adenosine Triphosphate
AQP	Aquaporin
Aβ	beta-amyloid
BBB	Blood-Brain Barrier
BCA	Bicinchoninic Acid
BcL	B-cell Lymphoma
BIN1	Bridging Integrator 1
CAA	Cerebral Amyloid Angiopathy
CD2AP	CD2-associated Protein
CD33	Cluster of Differentiation 33
CNS	Central Nervous System
DAPI	4,6-diamidino-2-phenylindole
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12

DMSO	Dimethyl Sulfoxide
ECM	Endothelial Cell Media
EDTA	Ethylenediaminetetraacetic Acid
EEA1	Early Endosome Antigen 1
ELISA	Enzyme-Linked Immunosorbent Assay
FAD	Familial Alzheimer's disease
FBS	Fetal Bovine Serum
FDA	Food and Drug Association
GI	Guanidine Insoluble
GS	Guanidine Soluble
HBMECs	Human Brain Microvascular Endothelial Cells
HBSS	Hanks Balanced Salt Solution
ICD	Intracellular Domain
IDE	Insulin Degrading Enzyme
ISF	Interstitial Fluid
IgG	Immunoglobulin G
kDa	Kilodalton
Km	Michaelis constant
KO	Knock Out
LDL	Low Density Lipoprotein
LRP1	Low Density Lipoprotein receptor-related protein 1
LXR	Liver X Receptor
MCI	Mild Cognitive Impairment
MMP	Matrix Metalloproteinase

MMSE	Mini-Mental Status Exam
M-PER	Mammalian Protein Extraction Reagent
MRI	Magnetic Resonance Imaging
ND	Non-Demented
NEP	Neprilysin
NFTs	Neurofibrillary Tangles
NMDA	N-methyl-D-aspartate receptor
PBS	Phosphate Buffered Saline
PDGR	Platelet-derived Growth Factor Receptor
PFA	Paraformaldehyde
Pgp	P Glycoprotein
PICALM	Phosphatidylinositol Binding Clathrin Assembly Protein
PMSF	Phenylmethylsulfonyl Fluoride
PPARγ	Peroxisome proliferator-activated receptor gamma
PS1	Presenilin 1
PS2	Presenilin 2
Rab	Ras-related Proteins in Brain
RAGE	Receptor for Advanced Glycation End Products
RXR	Retinoid X Receptor
sAPPα	Soluble Amyloid Precursor Protein alpha
sAPPβ	Soluble Amyloid Precursor Protein beta
SEM	Standard Error of Mean
TGN	Transgolgi Network
TIMP	Tissue Inhibitor of Metalloproteinase

tPA	Tissue Plasminogen Activator
TREM2	Triggering Receptor on Myeloid Cells 2
Trk	Tropomyosin receptor kinase B
TYROBP	TYRO Protein Tyrosine Kinase-Binding Protein
TZD	Thiazolidinediones
WT	Wild Type
ZO	Zonula Occludens
α_2M	α2-macroglobin
α7nAChR	alpha 7 Nicotinic Acetylcholine Receptor

Chapter 1 : Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease associated with the build-up of beta-amyloid proteins (Aβ) in the brain and cerebrovasculature, in addition to the formation of neurofibrillary tangles (NFTs) comprised of a microtubule associated protein tau [1]. The accumulation of Aβ precedes both the neuroinflammatory and neurotoxic events associated with the disease, suggesting it may be a driving factor in the development of AD [2]. In addition, levels of soluble oligomeric Aβ in the brain correlate with the extent of neurodegeneration as well as the severity of the cognitive symptoms, indicating its involvement in the chronic deterioration associated with AD [3,4].

The majority of AD cases are sporadic and idiopathic, accounting for approximately 98% of the cases, with the remaining 2% occurring as result of autosomal dominant mutations [5]. AD is characterized by alterations in higher cognitive functions including: learning and memory, mood, loss of appetite, social behaviour, hyperactivity, and depression [6]. Currently, the clinical diagnosis is based on several criteria including the Mini-Mental Status Exam (MMSE), neurological, psychiatric examinations, medical history, and neuroimaging techniques, the latter of which has only recently become a viable method [7]. Despite the recent advances in the field of neuroimaging, especially in early diagnosis at preclinical stages, these approaches currently lack specificity and are mostly used in a clinical setting to rule out other similar conditions. Therefore, confirmation of AD diagnosis is still only possible with post-mortem pathological analyses based on the presence of extracellular Aβ plaques and Neurofibrillary Tangles (NFTs) which are located primarily in the hippocampus, amygdala and cerebral cortex [8,9]. It should also be noted that Aβ plaques and tau tangles are present in the brains of elderly non-AD individuals, albeit to a much lesser degree than in AD subjects [10].

1.1 Prevalence of Alzheimer's disease and current therapies

Worldwide, it was estimated in 2012 that there were over 34 million people currently effected by AD, and that the prevalence of the disease will triple by 2050 [11]. In developed countries with aging

populations, dementias, of which AD is the most common type, are of particular concern. This is due to their chronicity, the lack of effective treatments, the high caregiver burden, and the high personal and financial cost. Despite a concerted effort by the research community, a cure has yet to be identified and only palliative treatments with marginal effectiveness are currently utilised. Of the 413 clinical trials in phase I, II or III completed between 2002 and 2012, 99.6% of those failed, leaving only a 0.4% success rate [12]. There are currently only 5 medications which are prescribed for the palliative care of AD and primarily target cognitive and memory deficits. These include four acetylcholinesterase inhibitors (tacrine, rivastigmine, galantamine and donepezil) and one NMDA receptor agonist (memantine) which was the only FDA approved drug between 2002 and 2012. However, the improvements which occur as a result of these treatments only range from marginal to modest in cognitive assessments, and benefits to behavioural symptoms and day-to-day activities have demonstrated mixed results [13,14]. Even when administered as a combination therapy, donepezil and memantine only show statistically significant improvements in cognition which were considered clinically marginal [15]. The poor efficacy of existing treatments suggests a lack of understanding of both the aetiology and the pathological mechanisms driving the development of AD. What is clear, is the that therapies targeting the cognitive symptoms associated with AD have had limited success and new treatment strategies are necessary, particularly those that are disease modifying.

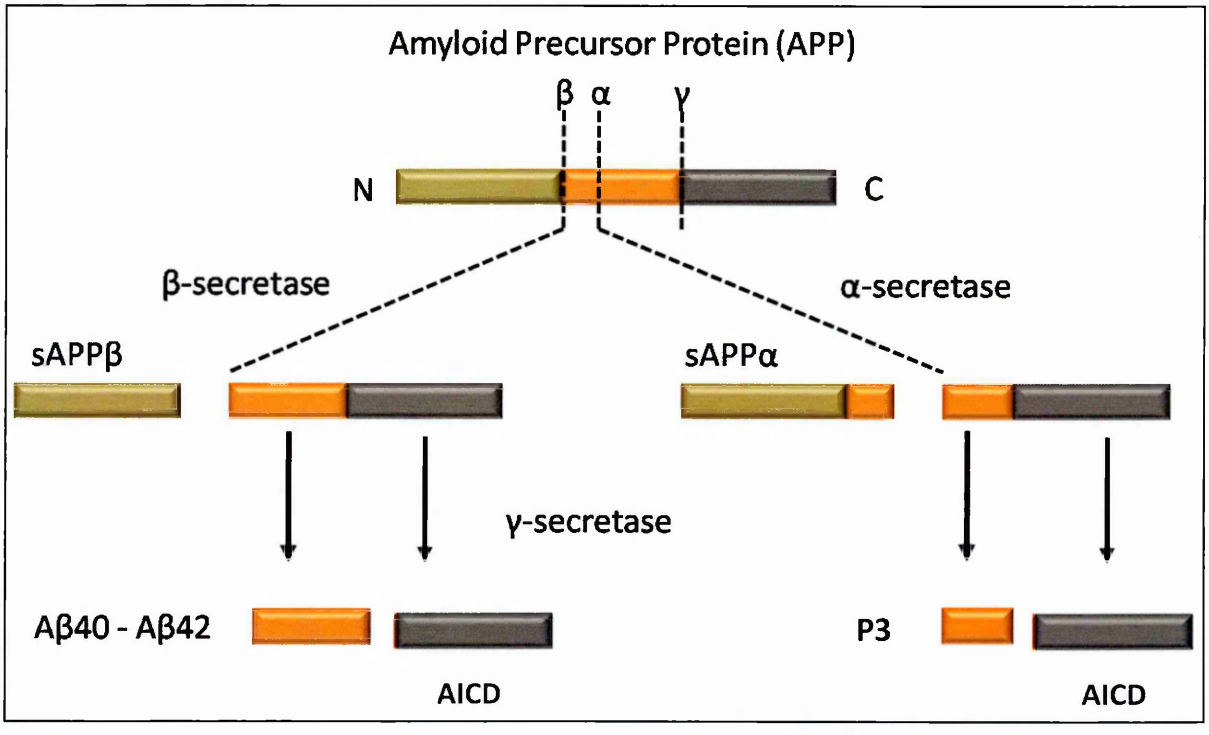


Figure 1.1 Processing of APP by the α-, β- and γ-secretases. Approximately 10% of APP enters the amyloidogenic pathway (left) which is driven by β-secretase cleavage of APP and results in the eventual production of Aβ. This initial step produces a sAPPβ protein and a fragment containing the remaining 99 amino acids in the APP sequence (C99). This C99 fragment is subsequently cleaved by the γ-secretase to produce an Aβ peptide whose size ranges from 38 to 42 amino acids in length, with Aβ40 proving to be the most common. The other fragment produced is the AICD which may have a role in transcriptional activation, although this role remains controversial. The remaining 90% of the APP processing is diverted down the non-amyloidodenic pathway by the α-secretase mediated cleavage of APP which occurs in the Aβ domain, thus preventing the generation of the Aβ peptide. The two fragments produced by α-secretase cleavage are the larger sAPPα and the smaller carboxy-terminal fragment (C83). The C83 fragment can undergo further processing by the γ-secretase to produce the P3 fragment, which currently has an unknown biological role, and the AICD.

Over the past decade, one of the major disease modifying approaches investigated for the treatment of AD has been to modulate the proteases involved in the production of Aβ. Aβ is produced from the amyloid precursor protein (APP), which is primarily expressed on neurons, by the β- and γ-secretases [16]. After the initial production of APP, it is trafficked to the cellular membrane where it is rapidly re-internalised and localised to the Golgi apparatus [17]. Here, it undergoes the amyloidogenic processing, eventually producing the Aβ peptides through the sequential cleavage of APP by the β- and γ-secretases. This process initially results in a soluble APPβ (sAPPβ) before further processing by γ-secretases which produces an APP intracellular domain (AICD) and an Aβ peptide (Figure 1.1)[16]. Aβ is subsequently trafficked into endosomes and is then released into the extracellular environment [18]. These Aβ peptides range in length, from between 32 to 43 amino acids due to the imprecise cleavage in the final γ-secretase mediated step, with Aβ40 proving to be the most common form [19]. Of the possible species produced, Aβ42 has the strongest tendency to aggregate and is thought to be the driving factor behind the formation of amyloid plaques and the neurotoxic events seen in AD [20].

1.2 The amyloid cascade hypothesis and familial Alzheimer's disease

While the pathophysiology of AD is incredibly diverse, the prevailing hypothesis for the aetiology of AD over the last 20 years has been the amyloid cascade . This theory was developed as a result of the identification of several mutations in the APP gene that caused overproduction and deposition of Aβ resulting in the formation of Aβ plaques. These mutations were found to cause the early onset familial AD (FAD) and, while cases remain rare in comparison to sporadic AD, they have nevertheless played an important role in establishing the central role of Aβ in AD, in addition to being vitally important for the development of AD animal models. Currently, more than 25 mutations in the APP gene have been identified that result in FAD as well as several other related hereditary diseases such as cerebral amyloid angiopathy (CAA) (http://www.molgen.ua.ac.be/ADmutations/). In addition to mutations in the APP

gene, several mutations have been identified in the γ-secretase subunits presenilin 1 (PS1) and presenilin 2 (PS2), many of which have also been integrated into animal models of AD. While the presentation of the pathology that occurs as a result of these mutations varies slightly, as does the level of penetrance, all result in the overproduction and deposition of Aβ. Interestingly, a mutation in the APP gene (A673T) was recently discovered that was found to protect individuals from AD by reducing the production of Aβ [21,22]. While this mutation remains rare, even compared to other FAD mutations, it further confirms the role of Aβ and its accumulation in the pathology of AD [23].

Even with the advances that the identification of these mutations allowed in the understanding of AD aetiology, the amyloid cascade hypothesis required multiple revisions over the years. More recently, it has been discovered that the expression of aggregated Aβ fibrils, which are a major component of the Aβ plaques, does not correlate well with the neuronal apoptosis and synaptic dysfunction seen in AD. While the aggregation of Aβ remains a prominent feature of AD, and is still one of the primary conditions required for a positive diagnosis of AD at autopsy [24], the soluble oligomeric species appear to be the main driver of Aβ-related toxicity [20,25,26]. Oligomeric Aβ is found both extra- and intra-cellularly and can translocate between the two environments [27,28]. While it is now well established that oligomeric Aβ accumulation results in a toxic cellular environment, the exact oligomeric structures and mechanisms by which Aβ toxicity is induced have not been conclusively demonstrated. However, the mechanisms that have been identified broadly fit into three categories: receptor-mediated neurotoxicity, cellular membrane dysfunction, and intracellular Aβ toxicity [29]. The receptor-mediated toxicity is thought to occur as a result of Aβ binding to several different receptor types. These include the NMDA receptors, where they interfere with long term potentiation induction and several downstream signalling pathways [30], and the α7 nicotinic acetylcholine receptor (α7nAChR), which has been shown to have particularly high affinity with Aβ and results in the internalisation of the receptor and the bound Aβ peptide [31,32]. The accumulation of intracellular Aβ oligomers has long been linked to AD, although the mechanisms by which this may cause neurotoxicity remain unclear. The strongest

evidence for its mechanism of action suggests the intracellular accumulation of Aβ may inhibit several pathways in the ubiquitin-proteasome system and may therefore impair the endocytic trafficking of neuronal receptors [33]. In addition to this, accumulation of Aβ has also been observed in mitochondria and was related to diminished enzymatic activity and efficiency [34]. Finally, Aβ oligomers are also associated with a deterioration in the plasma membrane integrity, which is associated with an increase in permeability and elevated intracellular calcium concentration [35,36]. There are several theories as to the exact mechanism by which this dysfunction occurs, but most well established theories propose that Aβ induces enhanced ion mobility through lipid bilayers or that cation-specific channels are formed by Aβ oligomers themselves [37–39].

1.3 Blocking production of Aβ as a therapeutic approach

As indicated above, inhibition of both the β- and γ-secretases with the aim of blocking the production of Aβ were highlighted as prime targets to reduce the levels of Aβ that excessively accumulate in the AD brain. However, both approaches have met with disappointing results and have either failed to reach their clinical endpoint or were stopped due to adverse reactions [40–42]. For example, the phase III clinical trial of the γ-secretase inhibitor semagecestat was halted after a pre-planned interim analysis which identified an increased incidence of skin cancer, gastrointestinal symptoms and worsening cognitive scores in the treatment group. While the cause of these side-effects are as of yet unknown, it has been suggested that it may be as a result of altered processing of the Notch signalling pathway which is required in several cell fate decisions. However, this has yet to be conclusively demonstrated and there are several other proteins that are associated with γ-secretase function that could result in some of the observed off-target effects [43–45]. Similarly, targeting the β-secretases via small molecule inhibitors has also proved challenging. This is thought to be at least in part due to the relatively large catalytic site responsible for the β-cleavage of APP. *In vitro*, inhibition of β-secretases can easily be

achieved with larger inhibitor molecules. Unfortunately, these inhibitors have not successfully translated into *in vivo* paradigms due to difficulties arising from bioavailability and penetration of the blood-brain barrier (BBB) [46]. In addition, off-target effects have often been reported with β-secretase inhibitors [47,48]. For example, the β-secretase inhibitor RG7129 was withdrawn from development after a phase I trial in 2013, most probably as a result of high levels of liver toxicity, although an official report was never released [48]. However, despite these failures, there are several β-secretase inhibitors still under investigation, although the attrition rate for the potential treatments remains high [40].

1.4 Antibody based therapeutic approaches

Another avenue of therapeutic intervention in AD has been through the use of Aβ directed immunotherapy. Similar to the targeting of the β- and γ-secretases, both active immunisation (injection of a non-toxic, non-fibrillogenic modified Aβ peptide) and passive immunisation (injection of 'pre-made' monoclonal anti-Aβ antibodies) were designed to reduce the levels of Aβ in the brain, albeit through difference mechanisms. For example, active immunisation aims to stimulate production of anti-Aβ antibodies in the periphery, acting as a peripheral sink for Aβ, whereas passive immunisation aims to increase clearance of Aβ by binding to Aβ in the brain and inducing Fc-mediated phagocytosis by microglia [49,50]. Initially, both active and passive immunisation approaches produced promising results in animal models [49,51]. However, clinical trials using the active immunisation approaches have shown little of no benefit [52,53]. In addition active immunisation was found to induce considerable side effects including the development of meningoencephalitis [52]. However, several of the current generation of active immunisation approaches have showed positive results in phase I trials, although none have yet published a successful outcome in phase II [54]. In comparison to the active immunisation route, passive immunisation remains a more active area of research, although the first generation passive immunisation antibodies also displayed little or no benefit to patients [55,56]. To date, multiple phase II

and III trials have been completed to varying degrees of success using passive immunisation. These include the phase III clinical trial utilising solanezumab, a humanised murine antibody directed against soluble Aβ species, which showed some significant improvements. However, this positive outcome was only evident in the neuropsychological tests of the patients with the mildest disease, suggesting that intervention in the early stages, or even prophylactic therapy may be beneficial [57]. Despite the more positive results from such trials, the passive immunisation approaches are also not without considerable side effects, with a number of patients developing what became known as amyloid-related imaging abnormalities (ARIA). These are comprised of two subsets and were originally detected through magnetic resonance imaging (MRI): ARIA-E, which is thought to represent vasogenic oedema and breakdown of the BBB, and ARIA-H which represents microhaemorrhages in the cerebrovasculature [58]. These side effects were found to be relatively common in some passive immunisation approaches, with all phases of the trials utilising the humanised monoclonal anti-Aβ antibody bapineuzumab observing increased ARIA associated with increasing dose of treatment [55,59,60]. In response to this, MRI imaging techniques are now widely used to assess safety outcomes in clinical trials and to screen for ARIA side effects [61]. Perhaps the most promising passive immunotherapy currently undergoing clinical trials is the human monoclonal antibody, Aducanumab, which has demonstrated dose dependent reductions in amyloid deposition and slow in the decline of the MMSE scores [62]. Similarly to other passive immunisation approaches, ARIAs have been observed with Aducanumab treatment, especially in the higher doses and in the APOE4 genotype [62]. However, the indications from the Aducanumab trails remain positive and a large scale phase 3 trial is expected to conclude in 2022 (ClinicalTrials.gov identifier: NCT02477800).

1.5 Stimulating non-amyloidogenic processes as a therapeutic approach

In addition to the amyloidogenic processing of APP, it is also proteolytically shed by the α-secretase family of proteins resulting in a sAPPα fragment. There have been several proteases identified that are capable of facilitating cleavage of APP at the α-site, although in neurons it is likely that A disintegrin and metalloproteinase 10 (ADAM10) is the main constitutively active protease [63,64]. The benefits of processing APP through the α-secretases route are two-fold. Firstly, sAPPα peptides have been demonstrated to have both neuroprotective and proliferative functions such as supporting the proliferation of neural stem cells [65]. In addition, the cleavage site for the α-secretases is located in the Aβ domain of APP, essentially blocking the ability of β-secretases to initiate the amyloidogenic cascade of APP, thus precluding the production of Aβ. Strategies targeting the α-secretase pathway, with the aim of modulating the production of APP, have been slower to progress, possibly due to the research communities focus on the inhibition of β- and γ-secretases as well as the immunotherapy approaches. Despite this, animal models and *in vitro* studies utilising an α-secretase activator, bryostatin, have shown promising results and good tolerability in phase I trials [66,67]. As a result, one phase 2 trial using bryostatin, initiated in 2015, is currently undergoing recruitment for moderate to severe AD (ClinicalTrials.gov identifier: NCT02431468). Aside from large scale clinical trials, there are early indications that bryostatin may show efficacy due to results from a compassionate use trial in a single patient with a PS1 mutation resulting in very early onset dementia and expressive aphasia [68]. This patient demonstrated improvements in speech, increased attention focus and improved ability to swallow as well as a decrease in limb spasticity with treatment.

1.6 The use of genome wide association studies to identify new targets

In contrast to these targeted lines of investigation, some research teams are taking a more global approach to understanding the mechanisms that are contributing to AD. Within the last five years, the largest genome wide association study investigating the genetic underpinnings of the progressive cognitive decline witnessed in sporadic AD was completed [69]. This study, which had over 74 000 participants, identified 19 gene loci, 11 of which were newly associated with AD, in addition to the APOE locus. Of these loci, immune-inflammatory genes were overrepresented suggesting that modulation of the brain's resident immune system is also implicated in the pathology of AD. This study was initiated, at least in part, due to the discovery of a rare susceptibility variant of the TREM2 gene which was found more frequently in AD patients [70]. TREM2 encodes the triggering receptor on myeloid cells 2 protein, which together with the TYRO protein tyrosine kinase-binding protein (TYROBP) triggers activation of the immune response in macrophages and dendritic cells [71]. The Trem2 protein is involved in the phagocytic pathway in microglia which is thought to be protective and result in the removal of cellular debris in the CNS and clearance of Aβ peptides in AD. In support of this, detection of activated microglia is well established as a marker for AD pathology in animal models. However, it remains unclear if this activation of microglia is a driver of pathology or simply a reaction to the inflammatory environment. There is some evidence to suggest that the activity of microglia, while initially anti-inflammatory may switch to a pro-inflammatory state after chronic exposure to Aβ peptides, resulting in reduced uptake of Aβ and increased production of pro-inflammatory cytokines and reactive oxygen species [72–74].

1.7 Mechanisms for the elimination of Aβ from the brain

Despite a concerted effort by both the research community and pharmaceutical companies to address the unmet need for treatments of AD, none of the current therapeutic avenues have, as of yet, reached an acceptable clinical outcome. Therefore, it is important to consider other strategies targeting alternative mechanisms that may be leveraged to modify AD progression. Recent research has suggested that the accumulation of Aβ may be due to a dysfunction of the Aβ clearance mechanism in the brain, and not through elevated or excessive production of Aβ [75]. Several mechanisms have been identified that contribute to the removal of Aβ from the brain which broadly fit into three distinct categories: enzymatic degradation, fluid/bulk flow clearance, and clearance through the BBB.

1.7.1 Enzymatic degradation

Enzymatic degradation of Aβ occurs both intra- and extra-cellularly by a diverse array of proteases. The extracellular degradation of peptides in the ISF is mainly attributed to the enzymatic activity of proteases secreted by astrocytes whereas the intracellular degradation occurs after internalisation of proteins into microglia and astrocytes. Once internalised, degradation occurs in several cellular compartments such as the endosomal and lysosomal compartments [76]. Of the proteases involved, neprilysin (NEP) and insulin-degrading enzyme (IDE) are perhaps the best characterised and have been found to show reduced expression in the hippocampus with age [77–79]. In addition NEP protein levels, mRNA and activity were all found to be reduced in AD when compared to normal controls [80]. The involvement of NEP in AD has been further studied in animal models of AD, with mice over-expressing NEP in neurons showing significantly lower levels of Aβ in the brain and no formation of Aβ plaques when compared to controls [81]. Interestingly, increasing expression of NEP in the periphery also appears to reduce the levels of Aβ in the brain [82]. However, modulation of NEP activity or expression may impact multiple cellular processes, particularly those involved in the regulation of the

cardiovascular system [83]. Moreover, high levels of NEP have been linked to congestive heart failure [84]. As a result, there is currently a phase III clinical trial investigating the viability of the NEP inhibitor, LCZ696, as a treatment for congestive heart failure (ClinicalTrials.gov identifier: NCT01920711). This suggests treatments that would result in a systemic increase in the expression of NEP, with the aim of facilitating the clearance of Aβ from the brain in AD may be not be a viable strategy as a result of the potential side-effects in the cardiovascular system. Regardless, this remains an area of active research and modulating cell specific expression of NEP remains a viable therapeutic target for increasing Aβ degradation [85].

1.7.2 Bulk flow clearance from the brain

Broadly speaking, there are two main fluid based clearance mechanisms in the brain, consisting of peri- and paravascular clearance pathways, both of which are capable of removing Aβ from the brain. The perivascular drainage pathway relies on perivascular arterial spaces which is are CSF-filled cavities that surround the small penetrating cerebral arterioles. These anatomical structures collapse on cardiac arrest and perivascular drainage stops, suggesting that vascular pulsations may be the motive force for this mechanism of clearance [85–87]. Studies have demonstrated that tracers injected into murine brains were found to rapidly colocalise with the vascular basement membranes in capillaries and arteries where they then flow in a opposite direction to the blood flow towards the subarachnoid space [86,88]. Solutes, such as Aβ, are then ultimately cleared from the brain via the cervical lymph nodes [89]. In CAA, is several structural changes in the vasculature, including loss of the smooth muscle around arterioles, thickening of the basement membrane and deposition of Aβ in the parenchyma are observed [90–92]. It occurs in a significant portion of the elderly population and in over 80% of AD patients [93,94]. The presence of CAA in animal models of AD was found to result in reduced perivascular drainage which has been hypothesised to result in a feed-forward process whereby Aβ deposition promotes further Aβ deposition in the vasculature, further dampening the perivascular drainage of solutes [95]. However, the perivascular clearance of Aβ is believed to be approximately 6-fold slower

than the transport into the blood via the BBB, suggesting that dysfunction in this clearance pathway may not be as impactful [96]. Regardless, chronic reductions in the perivascular drainage of Aβ from the brain are probably still a contributing factor to the reduced total clearance of Aβ witnessed in AD.

In contrast to perivascular drainage, paravascular (or glymphatic) clearance relies on the convective bulk flow of ISF away from arterioles to veins. This has been shown to be driven, at least in part, in part by the aquaporin 4 (AQP4) water channels expressed in astrocytic end feet, with AQP4 KO mice showing approximately 70% reduction in removal of solutes from the brain [97]. The first stage of paravascular clearance requires CSF influx into the periarterial space which occurs in the same direction as blood flow. Here the water from CSF drains through the AQP4 channels and enters into the brain parenchyma before the pressure gradient allows exit of fluid from the brain via AQP4 channels at the venous side. Waste then recirculates into the CSF or is cleared via the lymphatic systems [98]. With regards to AD, it was demonstrated that mice lacking astrocytic AQP4 had significantly reduced clearance of Aβ from the brain [97]. In addition, paravascular drainage and AQP4 expression it has also been shown to be change with age, indicating it may be a contributing factor for the risk of developing AD [99]. However, while it has been reported that disruption of paravascular drainage reduced Aβ clearance by approximately 55%, it was also shown that Aβ was cleared from the brain at a significantly faster rate than a comparable sized dextran [97]. This suggests that there is a selective clearance mechanism in the brain that specifically targets Aβ over molecules of the same size.In support of this, it has previously been shown the Aβ is cleared from the brain at rates far higher than can be attributed so bulk flow mechanisms, indicating other mechanisms, such as active or receptor mediated clearance at the BBB, may be a more prominent clearance route [100].

1.7.4 Clearance of Aβ through the BBB and LRP1

One of the main routes of Aβ elimination from the brain is through the BBB, accounting for at least 25% of total Aβ clearance [100,101]. The reduced BBB clearance and accumulation of Aβ in the AD brain is, at

least in part, believed to occur as a result of disrupted Aβ transporter function in brain capillaries [100,102,103]. Findings such as these has led to the development of the neurovascular hypothesis of AD which has been steadily gaining support over the past decade [102,100,96]. The BBB is formed primarily by endothelial cells and pericytes, although it requires additional support from astrocytes and neurons to maintain its integrity and function. Collectively, these components are termed the neurovascular unit. The cross talk between different cells of the neurovascular unit helps regulate the permeability of the BBB as well as cerebral blood flow (Figure 1.2). In particular, pericytes appear to have a prominent role in maintaining BBB integrity, with reductions in cell number and the subsequent breakdown of the BBB strongly associated with the development of AD pathology [104]. In comparison to peripheral blood vessels, movement of molecules through the BBB is tightly regulated. Charged molecules and larger proteins such as Aβ generally have very poor permeability across the BBB, and require specific transporter mechanisms to allow movement in either direction.

While pericytes are involved in the regulation of the BBB, the actual paracellular barrier is formed by endothelial cells as a result of the presence tight junction proteins. The tight junction proteins consist of at least 40 different proteins and are located at the cell-cell interfaces [105,106]. The primary tight junction proteins are the occludin and the claudin proteins which are found at the interface proximal to the lumen of vessels. The claudins are a family of over 20 proteins form tight junction barriers through a claudin-claudin interaction. At the BBB, claudin-1, -5, -3 and -12 are expressed to varying degrees, with claudin-5 being the most prevalent [107,108]. A reduction in claudin-5 expression is associated with ischemic insult and exposure to Aβ peptides, with complete knockout of caludin-5 resulting in severe BBB disruption and changes in BBB permeability [109–111]. In comparison to the claudins, the role of occludin proteins in the formation of the BBB is not as clearly established. On one hand, studies have linked the expression of occludin with control of membrane integrity as measured by electrical resistance [112]. In addition, occludin degradation induced by viral or bacterial infection increases the permeability of in vitro BBB models [113,114]. However, occludin deficient mice display normal tight

junction functionality suggesting that expression of occludin may not be essential for the formation of the BBB changes, and that BBB function is not dependent on the occludin proteins [115,116].

The main adaptor proteins responsible for the anchoring both claudin and occludin to the cytoskeleton in endothelial cells are the zonula occludens (ZO)-1, -2 and -3 proteins which have been shown to be essential for the assembly of tight junction proteins [117,118]. ZO-1 in particular appears to be important, with ZO-1 KO mutations proving to be embryonically lethal as it is required for normal blood vessel formation in the yolk sac. This suggests that it may be an indispensible protein for the organisation of the endothelial tissue [119]. Furthermore evidence for the essential role of ZO-1 in barrier function was found *in vitro*, with KO of ZO-1 proving to be much more disruptive to barrier formation and function than depletion of any other tight junction proteins [120].

As a result of this tight junction barrier, larger molecules that are not readily permeable through the BBB, such as Aβ peptides, require specific transporters to facilitate clearance from the brain. There are several receptors that are capable of facilitating the transport of Aβ through the BBB. These include P-glycoprotein (Pgp) and class members of the low density lipoprotein receptor (LDL) receptors [121,121,122]. Although the relative importance of these receptors in the clearance of Aβ through the BBB is disputed, a recent study using endothelial specific KO mice identified that the LDL receptor, low density lipoprotein receptor-related protein 1 (LRP1) was responsible for approximately 50% of the total BBB clearance of Aβ42, suggesting that this may be the primary receptor responsible for the clearance of Aβ from the brain [123]. It should also be noted that while both LRP1 and Pgp expression has been found to be reduce with age, loss in LRP1 expression occurs at a much younger age. This suggest that LRP1 may the original driver for the reduced clearance of Aβ and its subsequent accumulation witnessed in AD, whereas reductions in Pgp expression may be a secondary pathology [124].

1.8 LRP1 mediated clearance of Aβ through the BBB

LRP1 is expressed on a variety of different cell types in the CNS, in addition to the endothelial cells, where it is responsible for the uptake of Aβ from the extracellular matrix for proteolytic degradation [125–128]. When expressed on the endothelial cells of the BBB, binding of Aβ to LRP1 at the abluminal (brain-side) of the BBB causes rapid clearance of Aβ through transcytosis across the BBB into the blood [100,129,130]. This rapid transcytosis has been demonstrated *in vivo,* where the rate in which Aβ was cleared through the BBB was found to be far higher than the rate in which it is transported by bulk flow, implicating receptor mediated transport as a major mechanism for eliminating Aβ through the BBB [96]. While LRP1 is not the only member of the LDL receptor family that is capable of binding Aβ and facilitating transcytosis, it is thought to be the main receptor responsible for the endocytosis of ligands due to its faster rate of internalisation. For example, the very low density lipoprotein receptor (VLDLR) and LRP8 receptors from the LDL family internalise ligands at a $t_{1/2}$ of roughly 8mins, whereas LRP1 has a $t_{1/2}$ of 0.5mins [131]. After the rapid internalisation of the LRP1 and ligand complex, the ligands are dissociated from LRP1 in the early endosomes and the receptor is subsequently recycled back to the cell surface [132]. The definitive evidence for the importance of LRP1 in the transport of Aβ across the BBB was recently obtained by a group led by Storck et al. who developed conditional endothelial LRP1 KO mice in a FAD mouse model. These mice displayed a accelerated AD phenotype compared to FAD mice expressing endothelial LRP1, with significant reductions in Aβ-BBB transit, increased soluble Aβ in the brain, and deficits in memory and spatial learning [123]. This study demonstrates that reduced BBB clearance of Aβ is sufficient to exacerbate an AD phenotype in an AD mouse model. In addition, it confirms the vital role which LRP1 plays in clearance of Aβ through the BBB and the consequences that arise when this process is disrupted [123]. However, it remains unclear whether the loss of LRP1

expression in isolation is sufficient to induce an AD phenotype in humans without the increased production of Aβ found in animal models of AD.

LRP1 is produced as a precursor protein which is cleaved into two subunits in the Golgi complex by furin-like endoproteases resulting in a 515kDa α- and a 85kDa β-chain [133]. After this proteolytic processing, the extracellular domain 515kDa fragment is non-covalently bound to the transmembrane 85kDa domain of LRP1 and is trafficked to the cell surface [134,135]. Interestingly, aside from the endothelial cells, LRP1 is also highly expressed in several cell types in the CNS where it is involved in cholesterol regulation of uptake of Aβ. These include the microglia and neurons where LRP1 plays a major role in the uptake of Aβ for subsequent degradation [126,136]. LRP1 expression is also particularly high in the liver where it is involved in the final hepatic clearance of Aβ out of the body [137]. Interestingly, LRP1 expression has been found to decrease with age and in AD, likely resulting in reduced clearance of Aβ from the brain and implicating altered LRP1 expression as an early AD pathology and even potentially an aetiology of the disease [124,138].

The surface expression of LRP1 and its ability to facilitate clearance of Aβ through the BBB is at least in part regulated by the ectodomain shedding of LRP1 by proteases at the cell surface. This results in the formation of a soluble 515Da fragment that loses the ability to transcytose Aβ but maintains its binding profile. As a result, soluble LRP1 is detected in senile plaques co-localised with Aβ [140]. This preserved binding profile of soluble LRP1 has also been recognised for its potential as a AD therapeutic, with administration of soluble LRP1 fragments in the periphery acting as a peripheral sink for Aβ, promoting the continuous removal of Aβ from the brain [130]. Under normal conditions, this circulating soluble LRP1 is believed to sequester approximately 70-90% of free Aβ while also facilitating its eventual removal from the plasma [130]. In AD patients, this peripheral soluble LRP1 appears to have reduced ability to bind Aβ peptides, potentially as a result of increased oxidation, and therefore is unable to prevent re-entry of Aβ to the brain through transport by the receptor for advance glycation end

products (RAGE)[141,142]. There is some evidence to suggest that the balance between the LRP1 mediated efflux of Aβ and Aβ influx as a result of RAGE may be altered in AD. For instance, under normal conditions, the expression of LRP1 appears to be primarily in the vasculature while RAGE is expressed at

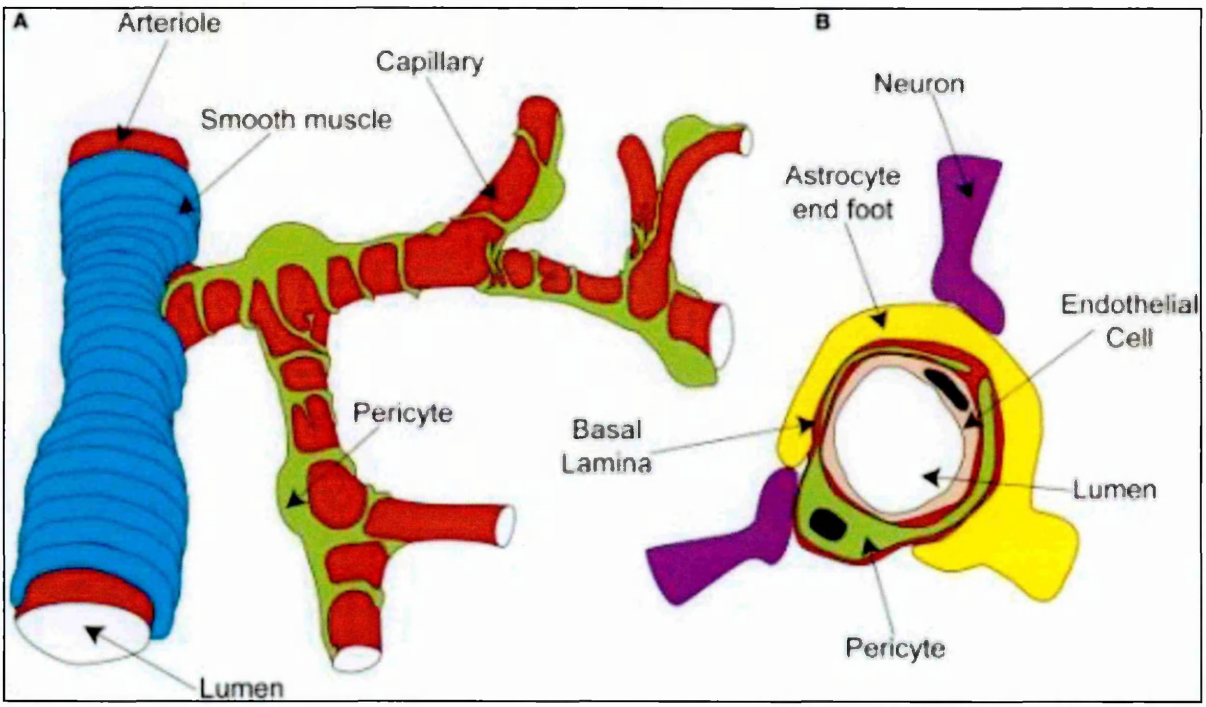


Figure 1.2 Organisation of the neurovascular unit. The structure and composition of the cerebrovasculature changes depending on the location. (A) The intracerebral arteries contain rings of vascular smooth muscle cells (blue) which encircle the endothelial cells and occupy most of the vascular wall. (B) After branching, brain capillaries are formed that lack the smooth muscle and are wrapped in pericytes (green). These pericytes envelop the branching capillaries and share a basement membrane with the endothelial cells (pink). The pericytes play multiple roles in the function and integrity of the neurovascular unit including the regulation of blood flow, secretion of extracellular matrix proteins and contribute to the clearance of cellular debris. Astrocytic end feet (yellow) help maintain a cellular link to the neurons (purple) in addition to maintaining BBB integrity, recycling glutamate to glutamine for neurons and maintaining homeostatic conditions in the brain. Image adapted with permission from Hamilton et al.[139].

a high density in the neurons [143]. However, in AD, the expression patterns are inverted, with LRP1 primarily expression in neurons and robust RAGE expression in the vasculature [143]. RAGE directed therapies have also shown some promise in ameliorating AD like pathologies and symptoms with anti-RAGE IgG increasing cerebral blood flow. In addition, mice overexpressing neuronal RAGE, which as previously discussed, is high in non-AD neurons, improved performances in special learning and memory tests. Despite these promising preclinical results and good tolerability of the RAGE inhibitor PF-004494700 in a phase I trial [144], the phase II trial was halted after interim analysis showed no benefit and treatment along with all future studies were abandoned [145]. Therefore, in the wake of these disappointing results, blocking LRP1 'sheddases', and therefore the inactivation of LRP1 through ectodomain shedding, remains an attractive avenue for therapeutic intervention. Some of the most prominent sheddases implicated in the ectodomain shedding of LRP1 include the α-secretase ADAM10 [146] and the matrix metalloproteinase 9 (MMP9) [147]. However, their physiological role in the shedding of LRP1 and AD pathology has yet to be conclusively studied.

1.9 Non-Aβ clearance roles of LRP1

Aside from the clearance of Aβ in the BBB, LRP1 is also involved in many other homeostatic and signalling processes in the brain. For instance, in forebrain specific knockout of LRP1 in mice display reduced dendritic spine densities and synaptic impairments, neurodegeneration in the absence of amyloid pathology, neuroinflammation and behavioural alterations [148]. LRP1 also plays a prominent role in cholesterol and apoE metabolism in neurons, with altered lipid and cholesterol uptake associated with exacerbated Aβ pathology in mice overexpressing APP in conjunction to a conditional KO of LRP1 in the forebrain [149].

In addition to its lipid metabolism and endocytic activity, LRP1 is also involved in the regulation of signalling pathways in response to ligand binding [150]. For example, the binding of tPA to LRP1 induces

Src family kinase activation and signalling through Trk receptors [151]. Reductions in Trk signalling have previously been detected in mouse models of AD where they were linked with deficits in memory based tasks [152]. In addition, altered Trk receptor expression has been found in neurons of AD patients suggesting that regulation of signalling through LRP1 may also be altered in AD [153]. The intracellular domain of LRP1 (ICD) has also been demonstrated to be involved in several signalling cascades and in the regulation of transcription, which requires prior proteolytic cleavage by the γ-secretase and translocation to the nucleus. For example, the LRP1 ICD has been shown to suppress the action of the interferon-γ promoter, resulting in the suppression of inflammatory responses [154,155]. In addition, the LRP1 ICD may have a role in the regulation of APP, as the LRP-ICD has been found to be highly colocalised with the APP transcriptional regulator, Tip60, in the nucleus [156]. It has therefore been suggested that basal levels of LRP1 shedding may be an important mechanism in the regulation of protein expression or cell signalling. However, it should also be noted that much of this work remains a speculative, and more information is required to conclusively prove that LRP1 shedding is directly involved in these events [157].

There is also an immerging role for LRP1 in the regulation of normal NMDA receptors function, with reductions in NMDA-dependent gene transcription and internalisation of AMPA subunits observed in LRP1 KO neurons *in vitro* [158]. LRP1 is also involved in the expression of the NMDA receptor subunit, NR2B, with mice expressing LRP1 containing a knock-in mutation in the NPxY domain required for the internalisation of the receptor, showing a reduction in NR2B levels. These changes in the NMDA receptor subunit composition is at least in part believed to be the cause of the altered behaviour and changes in spatial learning observed in LRP1 KO models. Finally, LRP1 deficiency has also been associated with altered vascular function and smooth muscle apoptosis via PDGF receptor mediated down-regulation of Bcl-2, further implicating it in the vascular pathogenesis of AD [159].

1.10 ApoE and Alzheimer's disease

While the genome wide studies in AD identified several risk genes associated with inflammation, the main genetic risk factor for AD is the presence of the APOE4 allele [160,161]. ApoE protein exists as a 34kDa glycoprotein which has three isoform variants in humans (apoE2, apoE3 and apoE4). Possession of one or two APOE4 alleles increases the chance of developing AD by 4- and 15- fold respectively when compared to APOE3 homozygous individuals [162]. In addition, AD patients and transgenic AD animals homozygous for APOE3 show reduced brain Aβ deposition compared to subjects homozygous for APOE4 [163,164]. As well as having an increased Aβ brain load, carriers of the APOE4 allele exhibit brain amyloid pathology as early as age 40, experience faster cognitive decline and have an earlier age of onset for AD [165,166]. Therefore, the APOE allele is clearly an important factor to consider when determining the driving pathologies of AD.

It has also been demonstrated that the apoE isoforms have differing affinities for Aβ, with apoE3 showing roughly a 20-fold increase in its association with Aβ as compared to apoE4 [167]. The ability of apoE to bind receptors and ligands appears to be dependent on its lipidation status, with poorly lipidated apoE demonstrating altered binding affinities to Aβ. This altered lipidation was associated with increased amyloidogenesis in mouse models of AD, suggesting that the lipidation status of apoE may play a role in Aβ pathology [168–170]. There are also some indications that the lipidation status is isoform specific and may therefore play a role in causing the isoform specific effects with regards to AD pathology and Aβ accumulation [171]. However, to our knowledge, no study has yet been carried out that assesses the impact of AD on the lipidation status of apoE (in either humans or animal models). As such the role of apoE lipidation has yet to be conclusively established. Interestingly, deletion or overexpression of the ATP binding cassette A1 (ABCA1), which is responsible for the expression and lipidation of astrocyte-derived apoE, results in increased or reduced deposition of Aβ respectively in AD mouse models [168,169,172]. Another matter to consider is the fact that early studies looking into apoE-

Aβ interactions utilised purified apoE that is in a non-lipidated and non-physiological form. These studies found inverse binding profiles to those published in later investigations that used apoE in its lipidated form [167,173]. Although this requires investigators to use a degree of caution when drawing conclusions from older references with an apoE component, it does indicate that the lipidation status of apoE may be an important factor to consider when trying to model AD.

1.11 ApoE and LRP1 interactions

One of the most closely associated molecules for the LRP1 receptor is the apoE protein. Interactions between LRP1 and apoE have been shown to been involved in complex array of processes including the regulation of Aβ, transcytosis of proteins, and the regulation cholesterol transport in neurons [174]. LRP1 and apoE also have several interactions that have been shown to be beneficial for the maintenance of vascular function. For instance, in lipoprotein metabolism, the interaction between LRP1 and apoE stimulates a signalling pathway that results in the initiation of several mechanisms that are protective against vascular disease [175]. In addition, LRP1 also plays a role in the regulation of apoE recycling. In human hepatoma cells, LRP1 facilitates the accumulation of apoE in early endosomal and recycling compartments, resulting in increased recycling and reduced degradation of apoE [176]. With regards to the transcytosis of Aβ, the apoE-LRP1 interactions appear to influence the rate at which Aβ is cleared from the brain to the periphery in an isoform specific manner [177]. However, it is unclear whether the presence of apoE is facilitative or inhibitory as several groups have identified conflicting results [96,100,178]. In these studies, it should be noted that experimental conditions which favour the most physiological expression of apoE, such as appropriate concentrations and a physiological lipidation status, appear to show that apoE has a facilitative influence on the clearance of Aβ through the BBB. Regardless of whether apoE acts in an inhibitory or facilitative manner when observing Aβ-BBB clearance, the reports are consistent in the fact that apoE4 isoforms always result in the lower levels of

Aβ cleared from the brain compared to the other two isoforms. Our previous studies suggest that apoE can have both facilitative and inhibitory effects in terms of clearance of Aβ through the BBB [179]. These studies identified that, when in complex with apoE, Aβ clearance is significantly reduced. This is in comparison to unbound apoE which appears to facilitate the clearance of Aβ through the BBB in an isoform specific manner [179]. In addition, evidence from autopsied brains appears to support the hypothesis that apoE is protective with regards to AD pathology. In this study, analyses of apoE expression in over 100 autopsied brains identified an inverse correlation between the apoE levels and brain Aβ load [180]. On this evidence, several therapies directed at increasing the expression of apoE were developed, such as the RXR agonist bexarotene and the LXR agonist TO90137. Both of these compounds have shown some success in animal models of AD, demonstrating the ability to increase the clearance of Aβ and improve performance memory related tasks [181–184]. However, contradicting results have been documented [185] and there are indications that this approach may have limited translational application due to systemic hepatomegaly as a result of treatment [186].

1.12 Aims

With the high rate of failure in AD clinical trials over the last decade, expanding the current mechanistic understanding of the underlying pathology of AD may not only unveil potential oversights that resulted in some of the recent disappointing clinical trials, but also identify potential new therapeutic avenues for the treatment of AD. As such, targeting the mechanisms involved in the regulation of LRP1 and its ability to remove Aβ from the brain has, as of yet, not been fully investigated. In addition, despite the strong association of the apoE4 isoform with the increased risk of developing AD, the mechanism by which it alters LRP1 function and its endocytic activity has not been conclusively demonstrated. Therefore, to address this, this thesis will investigate the role of LRP1 regulation in AD as well as the impact of apoE on the functions of LRP1. This will be investigated in the following aims:

1.To determine the impact of apoE on LRP1 shedding and the implications for Aβ BBB clearance

- Investigate the impact of Aβ on the shedding of LRP1
- Assess the influence of the apoE on the shedding of LRP1 and clearance of Aβ through the BBB
- Determine whether there is an apoE-isoform or AD dependent expression profile for LRP1 in the brain and cerebrovasculature
- Confirm the proteolytic activity of several of the known sheddases against LRP1

2. Assess the influence of apoE on the internalisation and sub-cellular trafficking of LRP1 and Aβ in brain endothelial cells

- Determine the impact of the apoE isoforms on the internalisation of LRP1 and Aβ
- Evaluate the impact of apoE on the localisation of Aβ in early and recycling endosomes

3. Investigate the impact of the LRP1 protease ADAM10 on the shedding of LRP1 and the implications on the clearance of Aβ through the BBB

- Analyse the impact of pharmacological inhibition of ADAM10 on the shedding of LRP1 and the clearance of Aβ through the BBB *in vitro*
- Assess the effect of ADAM10 on the clearance of Aβ through the BBB *in vivo* with ADAM10 endothelial specific knock-out mice
- Investigate the impact of ADAM10 inhibition on the shedding of LRP1, clearance of Aβ through the BBB, and the brain Aβ load in a mouse model of AD
- Evaluate the impact of the apoE isoforms on the activity of ADAM10 *in vitro*

4. Determine the influence of apoE on MMP9 and the shedding of LRP1 and clearance of Aβ through the BBB

- Analyse the impact of pharmacological inhibition of MMP9 on the shedding of LRP1 and the clearance of Aβ through the BBB *in vitro*
- Investigate the impact of the apoE isoforms on the activity of MMP9
- Assess the impact of MMP9 and shedding of LRP1 in the vasculature of APOE targeted replacement mice
- Evaluate the impact of pharmacological inhibition of MMP9 on the shedding of LRP1 and the clearance of Aβ through the BBB in APOE targeted replacement mice

Chapter 2 : ApoE dependent shedding of LRP1 and clearance of Aβ across the BBB

2.1 Introduction

The main avenue of exchange between the brain and the periphery for Aβ is the Blood Brain Barrier (BBB). In comparison to peripheral blood vessels, the movement of molecules through the BBB is tightly regulated. Polar molecules, amino acids and molecules larger than 400Da have very poor or no permeability across the BBB and require specialised transporters to facilitate transit [187]. One of the transport systems located in the BBB is the low density lipoprotein receptor-related protein 1 (LRP1), which is a member of the low density lipoprotein (LDL) receptor family. It is one of the receptors primarily responsible for the transport of Aβ across the BBB [123,125]. This receptor also has numerous functions including roles in the regulation of cholesterol transport, cell survival, blood coagulation, and synaptic transmission [128,188]. Binding of Aβ to LRP1 at the abluminal side of the BBB results in rapid transcytosis of Aβ across the BBB [100,129,130]. In addition, LRP1-mediated transport has also been shown to be an important route of elimination from the periphery via hepatic clearance of Aβ [137].

Previous studies have demonstrated that LRP1 expression is significantly reduced in the vasculature with age and in AD [138,189]. A diverse array of ligands have been found to interact with LRP1, including apoE [190], α_2-macroglobulin (α_2M) [151] and APP [191], all of which have been shown to be involved in the regulation of Aβ. In addition, Aβ is also well known to bind to the LRP1 receptor [192]. Restoring the expression of LRP1 to facilitate Aβ BBB clearance in the neurovasculature has been proposed as a potential therapeutic target for the treatment of AD. One promising avenue of study involves the use of thiazolidinediones (TZD) such as rosiglitazone or pioglitazone which have previously been shown to increase the expression of LRP1. TZDs function through activation of peroxisome proliferator-activated receptor-γ (PPARγ), which has long been in use for treatment of diabetes. Following studies demonstrating the epidemiological association between diabetes and AD [193], various TZDs were

investigated as potential AD therapies. Although any beneficial effect of rosiglitazone in AD was initially hypothesized to be due to its anti-inflammatory properties [194], it was later found that it may be due to up-regulation of LRP1 at the BBB. This effect has been demonstrated in several different cell types including adipocytes, hepatocytes, liposarcoma (SW872) as well as in human brain microvascular endothelial cells (HBMECs)[195–197]. In an AD-focused study, Moon et al. demonstrated that treatment of HBMECs with low dose rosiglitazone increased both the expression of LRP1 and the cellular uptake of Aβ [197]. However, they also demonstrated that high dose rosiglitazone did not show the same efficacy as the low doses, resulting in reduced expression of LRP1 and lowered uptake of Aβ. As such, simply altering the expression of LRP appears to be mechanistically complex, and may therefore not be the most viable approach increase Aβ clearance from the brain.

Cell surface expression and function of LRP1 is regulated, at least in part, by proteolytic shedding. This process yields a soluble LRP1 heavy chain fragment of 515kDa which includes the ligand binding sites, and an 85kDa light chain membrane bound fragment containing serine and tyrosine phosphorylation sites (Figure 2.1) [135,157,198]. Signalling through the serine and tyrosine induces several cellular processes including internalization of LRP1, cell division, survival and development[199,200]. Once LRP1 is cleaved, it loses the capacity to transcytose Aβ and may therefore cause reduced clearance of Aβ through the BBB. As demonstrated by Lui et al. and others, shedding of LRP1 can be induced by exposure to Aβ [201,202]. Although inactivated as a transcytosing protein when shed, soluble LRP1 maintains its high binding capacity and is thought to have several functions including activation of macrophages and acting as an amyloid sink in the periphery, the latter of which has been suggested as a potential target to reduce brain Aβ burden [130]. There are several species that have been identified as potential LRP1 'sheddases' including ADAM10 [146], tissue-type plasminogen activator (tPA)[203], and matrix metalloproteinase 9 (MMP9) [147]. However, their physiological role in the shedding of LRP1 and AD pathology has not been conclusively studied.

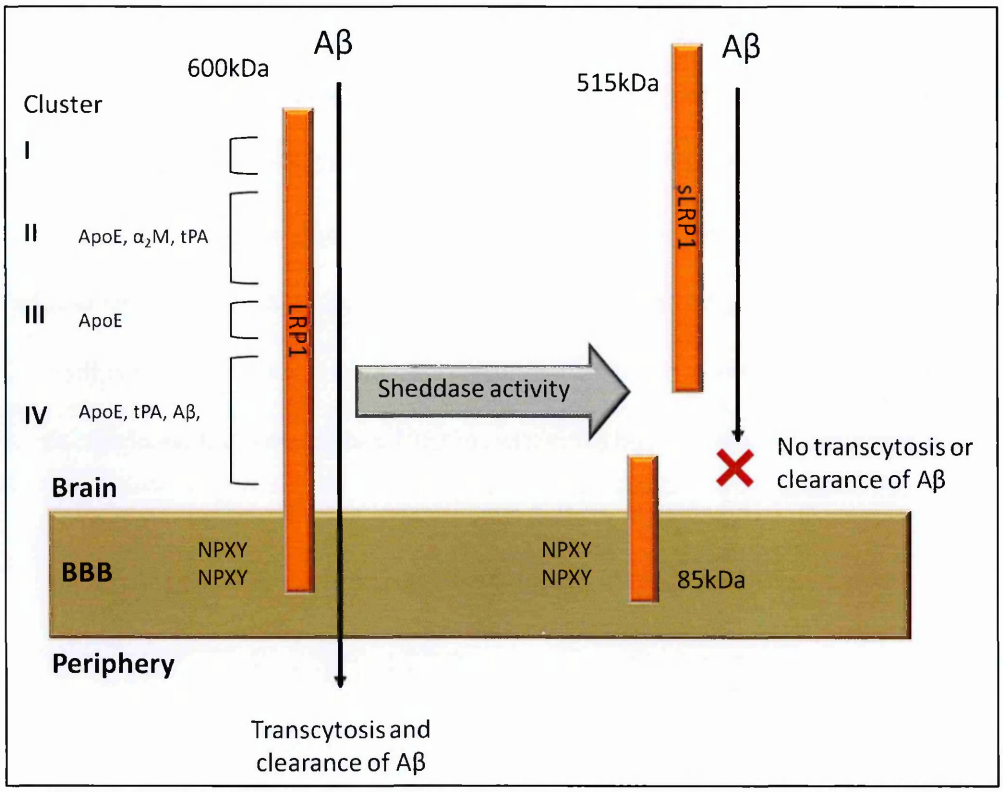


Figure 2.1: Full length LRP1 facilitates the clearance of Aβ through the BBB to the periphery. However, proteolytic shedding of LRP1 results in the soluble 515kDa fragment, and the cell associated 85kDa fragment which prevents the transcytosis of Aβ by LRP1. Multiple binding interactions with the extracellular cluster areas of LRP1 have been identified, many of which are associated with the regulation of Aβ and its production [204]. In addition, the intracellular NPXY tyrosine kinase and cytoplasmic domains have been shown to interact with a variety of adapter proteins, such as Shc, disabled and Fe65, which are involved in cellular trafficking and may also be involved in transcriptional modulation of its parent protein [157,205,206].

Another major factor affecting the BBB clearance of Aβ is the interaction with the Aβ chaperone apoE. ApoE is a 34kDa glycoprotein that exists as three common isoforms in humans (apoE2, apoE3 and

apoE4), with the APOE4 allele (encoding the apoE4 isoform) representing the strongest genetic risk factor for AD [160]. Possession of one or two APOE4 alleles increases the chance of developing AD by 4- and 15- fold, respectively when compared to APOE3 homozygous individuals[162]. It is well established that AD patients and transgenic AD animals homozygous for APOE4 show increased brain Aβ deposition compared to subjects homozygous for APOE3 [163,164]. One of the driving factors behind the increased accumulation of Aβ in AD is believed to the reduced clearance of Aβ from the brain, such as reduced clearance of Aβ through BBB [75,101].This process has also been shown to be influenced by the apoE isoforms, with APOE4-targeted replacement mice (APOE-TR) demonstrating reduced Aβ BBB clearance compared to APOE3-TR mice [179].

The ability of LRP1 to transcytose Aβ may be due to its binding interactions with the apoE isoforms (e.g. in complex with Aβ or unbound). Analysis of AD brains showed an increase in binding between apoE and Aβ compared to healthy controls, with samples homozygous for the apoE4 allele demonstrating the lowest levels of free apoE compared to other apoE isoforms [207]. These data are also supported by results from investigations in murine models, demonstrating that when Aβ is bound to apoE, Aβ transport across the BBB is dramatically reduced, with apoE4 mice showing the lowest levels of Aβ transport from the brain to the periphery [178]. However, as the majority of apoE appears to be unbound under physiological conditions, this suggests that apoE is likely facilitative in terms of Aβ clearance, although conflicting results have also been reported [208]. Experiments previously carried out by our group, as well as several others, support the facilitative role of apoE in Aβ clearance, with a complete absence of apoE significantly reducing the clearance of Aβ through the BBB and increasing the brain Aβ load [100,179,209,210]. Furthermore, analysis of the levels of apoE in over 100 autopsied brains identified an inverse correlation between the apoE levels and the Aβ load further supporting the beneficial role of apoE with regards to AD [180]. Although the mechanism for this altered apoE expression has not been established, it is possible that it may be linked to the expression of LRP1, which

has been shown to reduce the delivery of apoE to lysosomes, thus attenuating apoE degradation [176]. ApoE also appears to have altered binding to several proteins that have previously been implicated in the shedding of the LRP1 [211]. This suggests that while apoE may have a facilitative role in the clearance of Aβ, it also has potential to contribute to the pathology of AD as a result of its lipidation and isoform dependent binding to Aβ [170].

In light of the recent unsuccessful clinical trials for AD, an improved understanding of the relationship between LRP1 mediated clearance of Aβ and the apoE isoforms may pave the way for new avenues of therapeutic intervention to combat the cerebrovascular and BBB clearance abnormalities witnessed in AD. In particular, knowledge of the mechanisms that are involved in the regulation of LRP1 ectodomain shedding may be of importance for development of strategies that aim to increase the removal of Aβ from the brain and ultimately reduce the brain Aβ load. Therefore, this chapter will investigate the role of apoE in the ectodomain shedding and expression of LPR1 as well as its influence on the clearance of Aβ through the BBB using both *in vitro* and *in vivo* approaches.

2.2 Methods

2.2.1 Animals

Male APOE-targeted replacement (APOE-TR) mice (4-6 months of age) were purchased from Taconic Farms (USA). Mice carry one of the three human alleles (APOE2, APOE3, or APOE4) in place of the endogenous murine APOE gene [212]. These mice retain the endogenous regulatory sequences for the physiological expression of apoE while expressing the human apoE variants in the absence of mouse apoE. In addition, APOE knockout (APOE KO) mice were generated through disruption of the murine APOE gene, resulting in a complete absence of the mouse apoE protein [213]. Wild-type mice used in this study were of the same background (C57BL/6) as the transgenic apoE animals described above.

Mice were group housed in a temperature and humidity controlled environment on a 12hour light/dark cycle with free access to food and water. All the experiments involving mice were approved by the Institutional Animal Care and Use Committee of the Roskamp Institute.

2.2.2 Human cortex samples

Human cortex samples from the inferior frontal gyrus for each homozygous APOE genotype were acquired from multiple sources as follows: Banner Sun Health Research Institute (Sun City, AZ), the University of Maryland (College Park, MD) and the Mount Sinai NIH Brain and Tissue Repository (New York, NY). For full information regarding the funding sources and departments involved in these institutions, see the acknowledgments section. Demographic information is available in the Appendix (Table 9.1).

2.2.3 Preparation of Aβ42 peptides

Using a standard protocol to limit aggregation of Aβ42, both recombinant and fluorescein-Aβ42 were solubilised in 1,1,1,3,3,3-hexafluoro-2-propanol to acquire a monomeric/dimeric sample and to reduce formation of β-sheet structures as previously described [214]. All experiments involving Aβ42 utilised Aβ42 peptides prepared in this manner.

2.2.4 Collection and enrichment of human lipidated apoE

Glial cells expressing human apoE2, 3 or 4 isolated from neonatal mice were kindly provided by Dr LaDu (University of Illinois at Chicago) [215]. Lipidated apoE particles were collected and enriched from the mixed glial cultures as described in our previous work [202]. Briefly, human apoE expressing mixed glial cells were plated in 150cm^2 flasks (≈1.5 brains/flask) in DMEM/F12 (+10% FBS, L-glutamine (2mM), and 1% penicillin/streptomycin). Upon confluency, cells were washed and incubated with serum-free media for 72 hours. Glial-conditioned media was collected and centrifuged at 1000g for 3 min to remove any residual debris before concentrating (≈10x) using the Vivaspin 15R centrifugal concentrator with a molecular weight cut-off of 10 000 Da (Sartorius Stedim Biotech, USA). The resulting concentrate was

analysed for apoE content using a human apoE ELISA (MBL International Corporation, USA) and stored at -80°C until further analysis.

2.2.5 *In vitro* LRP1 shedding

Human brain microvessel endothelial cells (HBMECs) (ScienCell, USA) were seeded at approximately 50,000 cells per cm^2 into fibronectin-coated 6-well plates as previously described by our group [216]. When approximately 90% confluent, cells were treated with Aβ42 (2μM), both Aβ42 and one of the apoE isoforms (25ng/ml), apoE alone, or left as untreated controls before incubation for 48 hours at 37°C. Aβ42 was used at this concentration to minimise fibril formation which occurs with increasing concentration [217]. It has been previously been demonstrated that at concentrations lower than 10μM, fibril formation was not induced, despite incubation for 1 week under pro-fibril forming conditions [218]. Therefore, at 2μM Aβ42, fibril formation will be kept to a minimum and Aβ species should remain the soluble monomeric and oligomeric states. In addition, several inhibitors were tested to evaluate the role of the respective proteases in the shedding of LRP1. These were GI254023X (ADAM10 inhibitor at 1μM), SB-3CT (MMP9 inhibitor at 1μM) and a broad spectrum MMP inhibitor (1μM). All stock inhibitors were solubilised in 100% DMSO and concentrations of DMSO were <0.1% in all treatment wells. The levels of shed LRP1 were assessed in the media via LRP1 ELISA (Cedar Lane Labs, USA) as per the manufacturers' instructions. Additionally, a Lactate Dehydrogenase (LDH) assay (Roche Diagnostics, Germany) was carried out as per the manufacturers instruction to assess whether Aβ or a positive control, tPA, was inducing cytotoxicity in the HBMECs. As a positive control in the LDH assay, tPA at 10nM was included as it is a well established inducer of endothelial stress [219,220].

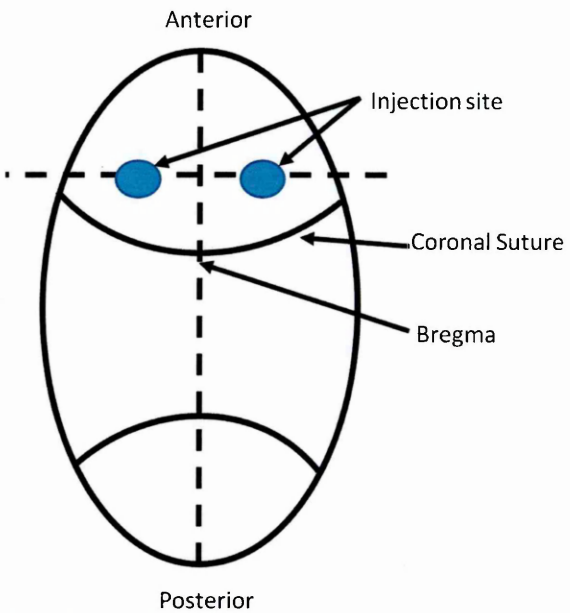


Figure 2.2: Dorsal view of mouse skull indicating injection sites for bilateral Aβ42 injections.

2.2.6 Brain LRP1 shedding and Aβ BBB clearance *in vivo*

To examine the effect of Aβ on the shedding of LRP1 *in vivo*, stereotaxic intracranial injections of Aβ42 were performed as previously described [202,221]. Briefly, male APOE-TR or APOE KO mice (4–6 months of age) were anesthetized via inhalation using a 4% isoflurane/oxygen mix. While under anesthesia, the mice were injected bilaterally with 3µl of vehicle (100% DMSO) or 1mM human Aβ42 into the caudate putamen of each hemisphere of the brain (0.5mm anterior to the bregma, 2 mm lateral to the midline, and 3 mm below the surface of the skull) with approximately 1 minute between the bilateral injections (Figure 2.2). Ten minutes after the second intracerebral injection, the mice were euthanatized. Upon sacrifice, all mouse brains were collected (minus the cerebellum) and the outer vessels and meninges were removed using a dry cotton swab [222]. All tissue samples were immediately snap frozen in liquid nitrogen and stored at −80°C.

2.2.7 Brain fraction isolation

Brain fractions were isolated from mouse brains (minus cerebellum and meninges) and human cortex samples (from the inferior frontal gyrus) using a technique previously described by our group [202]. Briefly, using a Dounce homogenizer, the tissue was homogenized in Hank's Balanced Salt Solution (HBSS) on ice. A sample was collected to represent the whole brain homogenate and diluted 1:1 in lysis buffer (M-PER + 1%EDTA +0.2%PMSF (Thermo Scientific, USA)) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, USA). The remaining homogenized tissue was diluted 1:1 with 40% dextran followed by centrifugation at 6000g for 15 minutes at 4°C. This resulted in a pellet in the base of the tube (cerebrovasculature) and a diffuse mass at the top (parenchyma) which were separated by a clear dextran solution (soluble fraction) (Figure 2.3). The parenchymal pellet was removed with a cell scraper into a separate tube with the soluble fraction and diluted 1:1 with ice cold HBSS. The parenchymal pellet was collected with lysis buffer following centrifugation at 6000g for 5 minutes. The soluble fraction was centrifuged one final time to remove any remaining debris. All samples were immediately stored at -80°C until further analysis by LRP1 ELISA (Cedar Lane Labs, USA). Demographics for the human samples can be found in the Appendix (Table 9.1).

2.2.8 Statistical analysis

Where appropriate, significance values were obtained through one-way or two-way ANOVA followed by Tukey *post-hoc* analysis (GraphPad Prism 5, GraphPad Software Inc., USA). Otherwise, where only 2 groups were compared, unpaired t-tests were utilised. All n values represent technical replicates.

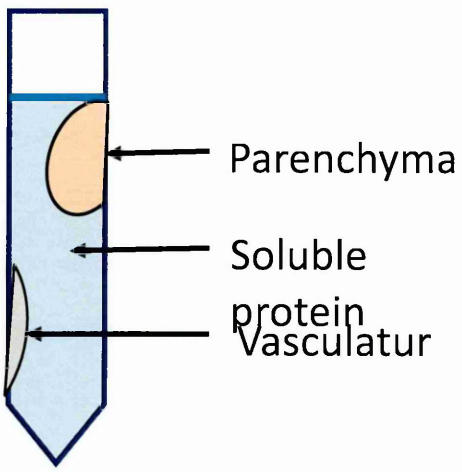


Figure 2.3: Schematic of the brain fraction separation after the initial centrifugation in 20% dextran for 15 minutes at 6000g with a fixed angle rotor.

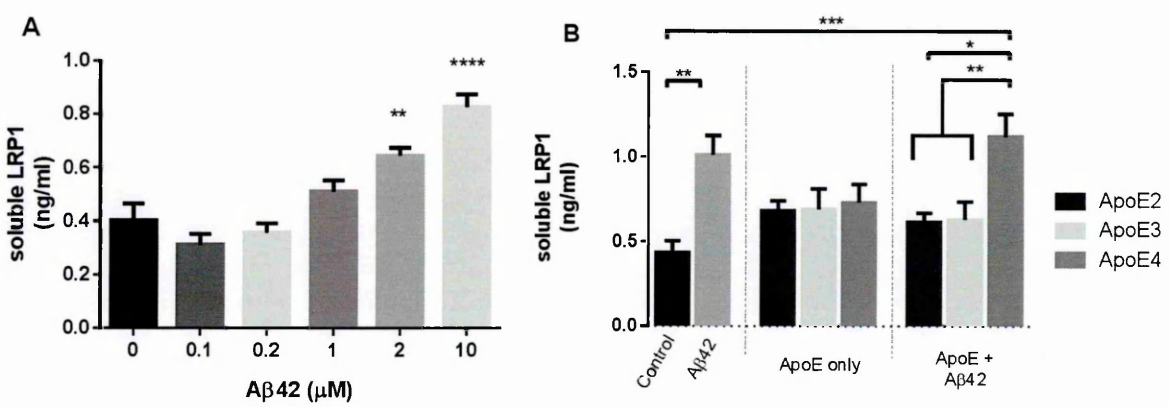


Figure 2.4 : LRP1shedding in HBMECs following exposure to Aβ42. (A) HBMEC monolayers were exposed to human Aβ42 (0-10μM) for 48 hours at 37°C. Shedding of LRP1 was dose dependent, with the highest levels of soluble LRP1 detected at 10μM Aβ42. (B) Co-treatment of HBMECs with apoE (25ng/ml) demonstrated protective effect of the apoE2 and apoE3 isoforms on the shedding of LRP1. In comparison, co-treatment with the apoE4 isoform did not alter the level of soluble LRP1 when compared to cells treated with Aβ42 only. LRP1 shedding was assessed by examining LRP1 content in the extracellular media using an LRP1 ELISA. Values represent mean ± SEM (n = 5) and are expressed as ng of extracellular LRP1 per ml of media. *p<0.05; **p < 0.01; ****p<0.0001 as determined by one-way ANOVA followed by Tukey's *post-hoc* analysis.

2.3 Results

2.3.1 *In vitro* shedding of LRP1 after Aβ42 and apoE exposure

To determine the effect of Aβ exposure on the shedding of LRP1, we exposed HBMECs to various concentrations of Aβ42 for 48 hours. A concentration dependent increase in the production of soluble LRP1 was detected in the media with statistically significant increases at concentrations higher than 2μM Aβ42 (Figure 2.4A). The LDH assay carried out on the cell media from this study identified an increase in cytotoxicity in Aβ42 treated cells at concentrations higher than 2μM after 48 hours. However, Aβ42 treated cells showed significantly lower LDH than the tPA positive control (60% reduction) (Appendix:

Figure 9.1). In addition, at the same concentration of tPA, the levels of soluble LRP1 were not significantly increased, suggesting that an increase in cell toxicity was likely not a primary factor in the generation of soluble LRP1. The influence of apoE on the shedding process was also assessed and revealed an apoE isoform-specific effect on the generation of soluble LRP1 (Figure 2.4B). Treatment with apoE alone produced a modest increase in soluble LRP1 which did not reach statistical significance when compared to untreated controls. Co-treatment of cells with Aβ and either apoE2 or apoE3 protected LRP1 from Aβ-induced shedding with significantly lower levels of soluble LRP1 compared to Aβ treatment alone. In comparison, levels of soluble LRP1 in the apoE4 and Aβ co-treated cells were significantly higher than the apoE4 only exposures.

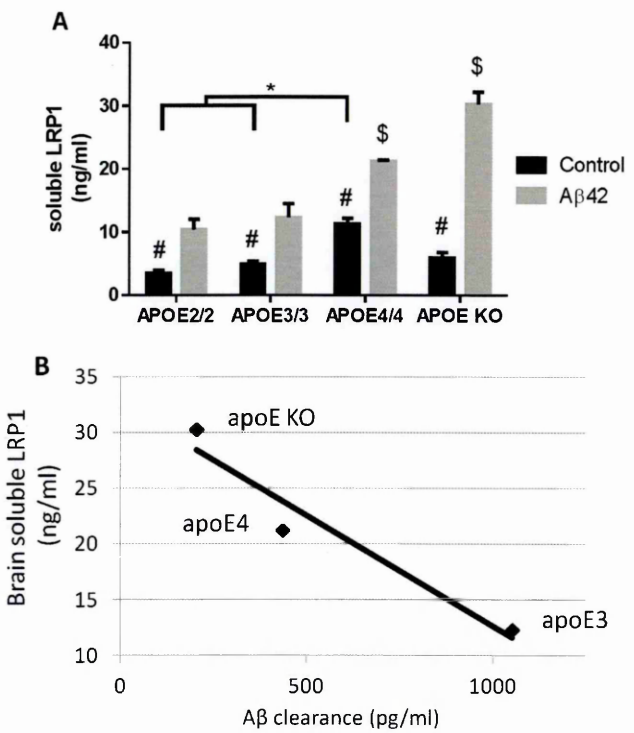


Figure 2.5 : Clearance of Aβ42 through the BBB and shedding of LRP1 following intracranial injection of human Aβ42. (A) APOE-TR and APOE KO mice demonstrated an increase in the shedding of LRP1 after intracranial injection. The APOE4 mice demonstrated the highest levels of shed LRP1 in both treated and naive mice when compared to the other treated and naive APOE genotypes. (B) When combined with data previously generated by our lab, an inverse correlation between the shedding of LRP1 and the clearance of Aβ through the BBB was evident (R^2= 0.94, $p<0.05$) [179]. LRP1 shedding was assessed by examining LRP1 content in the extracellular media or soluble brain faction using an LRP1 ELISA. Values represent mean ± SEM (n = 5) and are expressed as ng of extracellular LRP1 per ml. *$p<0.05$; $^{\#}p<0.05$ in the same genotype, $^{\$}$ $p<0.05$ compared to all others in treatment group. Significance determined by one-way ANOVA followed by Tukey's *post-hoc* analysis or by Pearson correlation analysis.

2.3.2 *In vivo* shedding of LRP1

To assess the influence of apoE on the shedding of LRP1 in an *in vivo* paradigm, mice were injected intracerebrally with exogenous human Aβ42 and the levels of soluble LRP1 measured after processing of the brain into fractions (e.g. homogenate, cerebrovasculature and soluble fractions). Similar to our *in vitro* study, exposure to Aβ42 induced shedding of LRP1 in all APOE-TR genotypes as compared to naive APOE-TR mice (Figure 2.5A). However, the level of soluble LRP1 in the naive APOE4 genotype was significantly higher than in either the naive APOE2 or APOE3 mice. In addition, the APOE4-TR mice treated with Aβ also displayed a higher level of soluble LRP1 than the other two APOE isoforms. Interestingly, the APOE KO mice showed the least protection of LRP1 from shedding after Aβ challenge, with significant increase in soluble LRP1 compared to all of the APOE genotypes. These data were combined with findings from Bachmeier et al. (2013) - performed prior to the commencement of this thesis)) which identified APOE genotype dependent differences in the clearance of Aβ after intracranial injection [179]. When levels of soluble LRP1 were assessed as a function of Aβ42 BBB clearance, an inverse correlation was observed between LRP1 shedding and Aβ BBB clearance (Figure 2.5B). Unfortunately, the clearance study did not include APOE2-TR mice. In its absence, APOE3-TR mice showed the highest level of Aβ42 BBB clearance and lowest levels of soluble LRP1. In comparison, the APOE-KO mice showed the lowest levels of Aβ BBB clearance and the highest soluble LRP1.

2.3.3 Expression and shedding of LRP1 in the human cortex fractions

To assess whether our *in vitro* and *in vivo* findings translated to the human population, we assessed the levels of LRP1 in cortex samples from the human inferior frontal gyrus. In the homogenate, total LRP1 was significantly higher in the ND group with approximately a 1.8-fold increase over the AD group (Figure 2.6A). When stratified by APOE genotype, APOE4/4 individuals in the ND group demonstrated significantly higher levels of total brain LRP1 compared to other ND APOE genotypes (Figure 2.6B). In addition, the levels of total LRP1 in the ND APOE3/3 and APOE4/4 group were also approximately 2-fold higher than those seen in the equivalent AD group. No significant change in the levels of soluble LRP1

were observed when assessed by disease state or when stratified by APOE genotype and disease state (Figure 2.6C). Expression of membrane associated LRP1 in the cerebrovascular fraction was approximately 2-fold higher in the ND samples when compared to the AD samples (Figure 2.6D). When stratified by APOE genotype, it was revealed that the levels of LRP1 were significantly altered by both the disease state and the APOE genotype (Figure 2.6E). While LRP1 levels in the vasculature were consistently higher in the ND compared to AD samples in all genotypes, with at least a 2-fold increase, significance was only reached in the APOE4/4 individuals. In addition, the APOE4/4 ND group showed significantly higher LRP1 expression in the vascular fraction than the APOE2/2 ND group.

2.3.4 Inhibition of LRP1 sheddases in HBMECs

Exposure of HBMECs to both Aβ42 and the MMP9 selective inhibitor SB-3CT or ADAM10 selective inhibitor GI254023X significantly reduced the levels of soluble LRP1 when compared to Aβ42 only treated cells (Figure 2.7). Levels of soluble LRP1 in SB-3CT and GI254023X treated cells were reduced to levels similar to those seen in the controls and were 50% and 60% of the Aβ treated cells respectively. The broad-spectrum MMP inhibitor lowered the levels of soluble LRP1 by approximately 25% compared to Aβ only treated cells but failed to reach statistical significance.

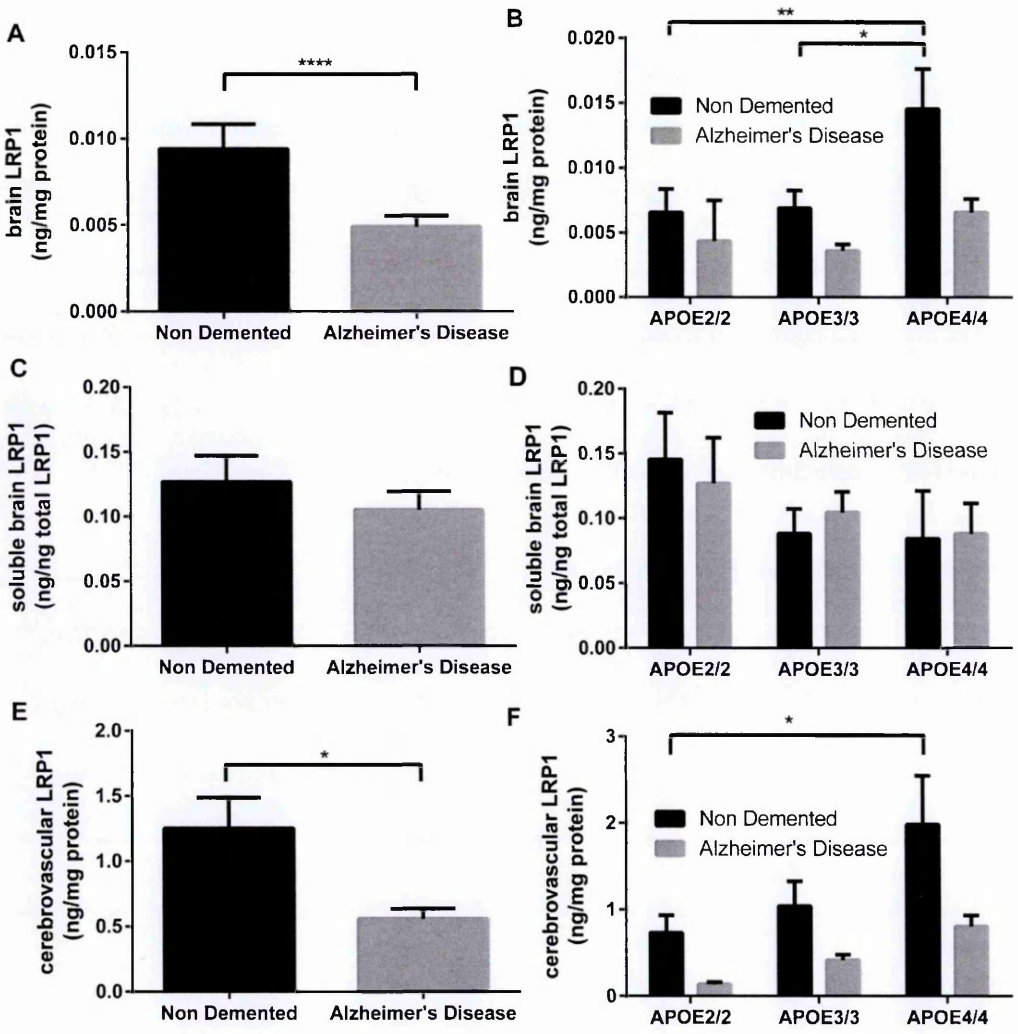


Figure 2.6 : Expression profiles of LRP1 in the human cortex and cerebrovasculature. (A) Expression of total brain LRP1 was significantly lower in individuals with AD when compared to non-demented controls. (B) This was found to be apoE dependent, with the apoE4 non-demented samples showing an elevated LRP1 expression compared to the other APOE genotypes. In addition, AD APOE4/4 samples showed significantly lower LRP1 expression when compared to the APOE4/4 non-demented controls. (C and D) No significant changes were detected in the levels of soluble LRP1 in the soluble brain fraction between non-demented and AD samples or when stratified by APOE genotype. (D) Levels of LRP1 in the cerebrovasculature were significantly lower in the AD samples when compared

to the non-demented controls. (E) This effect was apoE and disease dependent, with non-demented APOE4/4 samples showing the highest levels of LRP1 compared to the other APOE genotypes. In addition, AD APOE4/4 samples were significantly lower than the APOE4/4 non-demented controls. LRP1 expression was assessed by LRP1 ELISA. Values represent mean ± SEM and are expressed as ng of LRP1 per mg protein or ng soluble LRP1 per ng total LRP1. *p<0.05; **p<0.01; ****p<0.0001; #p<0.05 in the same genotype. Significance determined by Student's t-test or two-way ANOVA followed by Tukey's *post-hoc* analysis.

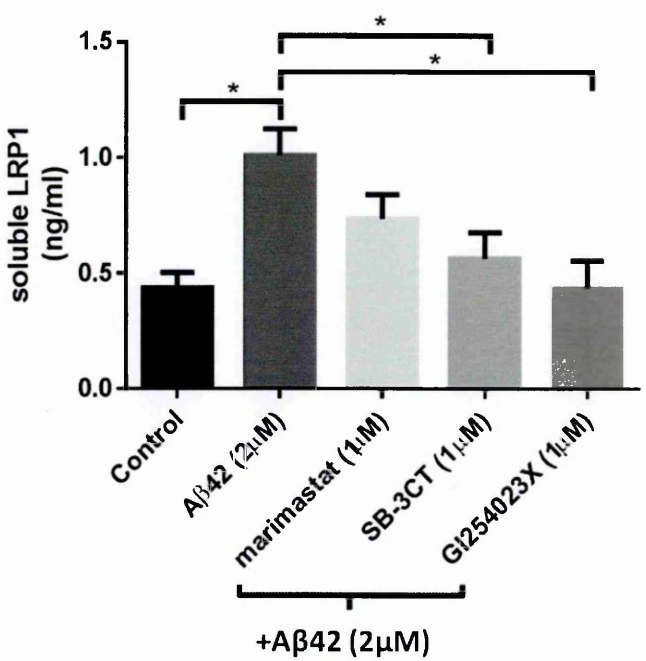


Figure 2.7 : Inhibition of LRP1 shedding in HBMECs. The shedding of LRP1 in HBMECS was reduced by treatment with a broad spectrum MMP inhibitor (marimastat), an MMP9 specific inhibitor (SB-3CT) and an ADAM10 specific inhibitor (GI254023X). LRP1 shedding was assessed by examining LRP1 content in the extracellular media using an LRP1 ELISA. Values represent mean ± SEM (n = 3) and are expressed as ng of extracellular LRP1 per ml of media. *p<0.05; as determined by one-way ANOVA followed by Tukey's *post-hoc* analysis.

2.4 Discussion

Transport of Aβ through the BBB by the lipoprotein receptor LRP1 is considered one of the main routes by which Aβ is removed from the brain. While most receptors in the lipoprotein family are capable of transporting Aβ, LRP1 endocytoses Aβ at a much faster rate, and is therefore considered the primary route [223]. In support of this, endothelial KO of LRP1 in mice results in a large reduction in the BBB clearance of Aβ and increased Aβ brain load [123]. Interestingly, it has previously been reported that levels of the functional membrane associated LRP1 is lower in individuals with AD [138]. However, the precise mechanism behind the reduced presence of LRP1 in AD is still not understood. Our current findings suggest the ectodomain shedding of full length LRP1, resulting in a non-functional soluble LRP1, may be one of the mechanisms by which expression is reduced. An understanding of what drives these mechanisms could pave the way to pharmacological interventions that increase the levels of full length LRP1 by blocking these shedding mechanisms, thereby reducing the brain Aβ load as a result of increased BBB clearance.

We demonstrate that Aβ42 induces shedding of LRP1 in both *in vivo* and *in vitro* conditions. In addition, when these data were combined with Aβ clearance data previously produced by Bachmeier et al. (2013) an inverse correlation was observed between the clearance of Aβ to the periphery and the levels of Aβ42 induced shedding of LRP1 in APOE-TR mice [179]. This suggests that the shedding of LRP1 may be one of the mechanisms responsible for the reduced Aβ clearance from the brain witnessed in AD and as a result of the APOE allele present.

The binding of apoE to Aβ has previously been shown to be an important factor in the clearance of Aβ through the BBB [179]. In comparison to unbound Aβ, Aβ bound to apoE was found to be cleared at a significantly slower rate. However, Verghese *et al.* demonstrated that under physiological conditions where the apoE protein is lipidated, the binding of Aβ to apoE is minimal, with only 6% of Aβ bound to apoE3 after 12 hours of incubation [208]. In addition, the rate in which Aβ and apoE3 associated slowed

significantly after the initial two hours and began to form a plateau. In our in *vitro* study investigating the shedding of LRP1, although the concentration of apoE utilised was slightly higher than those in the studies by Verghese *et al.*, we believe that the percentage of Aβ bound to apoE would remain minimal and we are therefore witnessing the effect of mostly unbound apoE on the clearance Aβ and shedding of LRP1. However, there are several limitations to the use of the lipidated apoE as a result of the methodology used obtain the protein. For instance, the apoE is in an enriched cell culture media, with size exclusion used to removal proteins smaller than 10kDa.Therefore the apoE solution contains proteins larger than this that may interfere with the mechanisms being investigated, such as the shedding of LRP1. However, the apoE only treatment in HBMECs showed no significant effect on the shedding of LRP1 and the effects observed appear to be as a result of an interaction between apoE and Aβ42. It is therefore unlikely that this mechanism is driven by another protein as the effects seen are specific to each of the APOE isoforms.

APOE4 remains the strongest genetic risk factor for developing AD and, despite its identification over 20 years ago, very little progress has been made towards establishing a definitive mechanism by which it appears to be less efficient than apoE2 or apoE3 in protecting from the development of AD. While several groups have investigated the expression of LRP1 and its relationship to total apoE levels, few have factored in the possibility of an isoform dependent relationship [224,225]. Of those that have accounted for the apoE isoform, conflicting results have been published detailing changes in LRP1 expression and mRNA levels depending on the apoE isoform present. For instance, both lower and higher levels of LRP1 expression has been reported in the APOE4 genotype in comparison to other genotypes [226,227]. Our investigation indicates that the APOE genotype influences expression of LRP1 in the cortex and in the cerebrovasculature. There was also a disease state dependent effect in levels of total LRP1, with the ND APOE4/4 sample showing by far the highest level. Analysis of the membrane associated levels of LRP1 in the vascular fraction, and therefore the most relevant in terms of BBB clearance of Aβ, revealed similar results to the total LRP1 levels, with both APOE genotype and disease

dependent effects. In all cases, the level of LRP1 in the vasculature was higher in the ND samples. When the results were pooled to assess disease state differences in all samples, the ND samples showed significantly higher levels of LRP1 in the vasculature. As membrane associated LRP1 is primarily responsible for the clearance of Aβ through the BBB, these data provide rationale for prior reporting that the clearance of Aβ is reduced by as much as 30% in AD [75]. However, one confounding factor which should be acknowledged when interpreting this data is that the ND APOE4/4 individuals had a lower average age than their AD counterparts. Therefore, some of the AD effects we witness may be due to this disparity in age or may even show a pre-AD phenotype in these individuals. It should also be noted that the expression of LRP1 in the cerebrovasculature appears to be much higher than the parenchyma when standardised to total protein, with approximately 100-fold more LRP1 per mg protein compared to the total homogenate.

Interestingly, the human samples showed no significant differences in the levels of soluble LRP1, suggesting that the ectodomain shedding of LRP1 may not be a pathological driver of AD. However, all our *in vivo* and *in vitro* analysis, as well as the work of others, suggests that shedding of LRP1 is exacerbated in an Aβ rich environment [146]. Therefore, it is possible that we were just unable to detect the changes in soluble LRP1, perhaps as a result of other proteins from the soluble fraction binding to LRP1 and masking the epitope. However, even if the levels of ectodomain shedding of LRP1 are not increased in AD or by the APOE genotype, shedding of LRP1 still occurs. In addition, maintaining higher levels of full length LRP1 appears to be beneficial, with ND individuals consistently demonstrating higher levels of membrane associated LRP1. Therefore, reducing the ectodomain shedding of LRP1 may still be a viable strategy as it could restore the levels of functional LRP1 and facilitate Aβ clearance regardless of the APOE genotype, especially in the vasculature where it would enable removal of Aβ to the periphery. In addition, restoring LRP1 expression may have other positive effects. Investigational therapies, such as bexarotene, which has been shown to reduce brain Aβ load and improve cognition, appear to be reliant on LRP1 expression to induce their beneficial effects [228]. In addition, several approaches have been

taken to increase the expression of LRP1 such as the PPARγ activator Rosiglitazone, which was capable of almost doubling the expression of LRP1 in HBMECs. However, the use of Rosiglitazone in a clinical setting is hampered by the relatively small therapeutic window for its efficacy [197]. Therefore, inhibition of the proteases responsible for the ectodomain shedding of LRP1 may be a more preferable approach. We demonstrate that inhibition of the sheddases ADAM10 and MMP9 successfully reduced the Aβ-induced shedding of LRP1 which has previously been reported by other groups [146,229]. While these are only preliminary results, and the actions of these proteins will be further investigated in Chapters 4 and 5 where their potential for pharmacological targeting will be assessed.

Peripheral soluble LRP1 has previously been demonstrated to play a major role as a peripheral sink for Aβ, binding between 70-90% of circulating Aβ [130]. It has also been demonstrated that the ability of soluble LRP1 to function in this manner may be reduced in AD, which may also contribute to the increased influx of Aβ into the brain via the RAGE receptors in the cerebrovasculature [130]. In this respect, the shedding of LRP1 and the presence of soluble LRP1 in the periphery may be advantageous in increasing clearance of Aβ reducing the brain Aβ load that would be impacted by inhibition of LRP1 sheddases. However, this sink mechanism is reliant upon on a functional BBB clearance mechanism for Aβ to enable it to reach the periphery. Therefore, the positive effects of the soluble LRP1 sink is rendered obsolete if Aβ is unable to be transported out of the brain. In light of this, we believe the restoration of BBB clearance mechanisms and prevention of LRP1 shedding in the endothelial cells should be a primary target for removal of Aβ to the periphery, and that it remains a viable approach to reduce the burden on Aβ on the brain.

In addition to the altered shedding and expression hypothesis outlined above, another explanation for the changes in transcytosis of Aβ through the BBB may be that the intracellular trafficking mechanisms responsible for the movement of Aβ are somehow altered or dysfunctional in AD. As apoE appears to alter the rate at which Aβ is transcytosed across the BBB, it is possible that these mechanisms are

sensitive to the apoE isoform present. Indeed, intracellular trafficking in various cell types has previously has been shown to be dependent on the apoE isoforms [230–232]. However, the bulk of this work has been conducted in neuronal and microglial cells. Therefore, Chapter 3 will investigate altered intracellular trafficking of Aβ in HBMECs as a result of the different apoE isoforms.

In summary, this chapter builds on the previously established hypothesis that reduced expression and function of LRP1 may be a contributing factor that drives the pathology of AD. As such, restoration of its clearance activity may prove beneficial in reducing the Aβ pathology. This chapter also links the expression of LRP1 and Aβ clearance through the BBB with the well known AD risk factor, APOE4. A better understanding of the mechanisms involved in the clearance of Aβ through BBB and the role in which apoE plays in this process may provide rationale for the failures of many AD clinical trials. For instance, previous clinical trials may have been ineffectual as a result of dysfunctional clearance of Aβ through the BBB masking the effects of the treatment, and not as a result of a lack of target efficacy. However, to effectively increase the expression of full length LRP1 at the BBB, a better understanding of the mechanisms that control its expression, such as the sheddases, will be necessary. Our *in vitro* and *in vivo* studies clearly demonstrate a strong inverse relationship between the ectodomain shedding of LRP1 and the clearance of Aβ through the BBB. In addition, we demonstrate that this relationship is apoE isoform dependent. We also identify apoE dependent changes in expression of LRP1 in the brain and the vasculature and found the LRP1 expression was reduced in both cases. To our knowledge, this is the first time AD and APOE dependent changes in the expression of LRP1 in the cerebrovasculature has been assessed. The results clearly indicate that APOE influences the expression of LRP1 and that the mechanisms involved in LRP1 regulation at the BBB may be dysfunctional in AD. As such, targeting these mechanisms may provide novel approaches to treating AD, particularly individuals with the APOE4 allele. The subsequent chapters will evaluate several mechanisms to further describe the observations presented here.

Chapter 3 : ApoE dependent uptake and subcellular localisation of Aβ

3.1 Introduction

Alzheimer's disease (AD) is characterised by the accumulation of amyloid β (Aβ) peptides both intra- and extra-cellularly. The clearance of Aβ has been shown to be reduced in AD by as much as 30%, suggesting that the mechanisms involved in the uptake, endocytosis and transcytosis of Aβ may be dysfunctional [75]. Transcytosis of Aβ through the BBB to the periphery requires three separate and distinct processes: (1) receptor mediated endocytosis of Aβ at the basolateral side of the BBB; (2) intracellular endocytic trafficking of the protein through the endosomal compartments and (3) the subsequent exocytosis of Aβ at the apical side resulting in the release of Aβ to the periphery. It has therefore been that suggested that dysfunctional endosomal trafficking may be a contributing factor to both the intracellular accumulation of Aβ [2,233].

Perturbed intracellular trafficking has previously been implicated as an early contributor to AD pathology. For instance, altered expression of critical proteins involved in endosomal sorting and maturation appear to correlate with the early signs of AD [234,235]. There is also evidence of defective endosomal function in later-stage AD and in AD animal models, with high levels of Aβ42 found in neuronal bodies resulting in synaptic dysfunction, deficits in long term-synaptic plasticity, and dystrophic neurons prior to the onset of Aβ plaque pathology [236–238]. Under non pathological conditions in neurons, Aβ is generated intracellularly at the endoplasmic reticulum where it is then transported to the trans-Golgi network (TGN) before being introduced into the endosomal system and finally being released into the extracellular environment [239–241]. In AD, it is believed the build up of intracellular Aβ may be a result of increased Aβ retention due to defective intracellular trafficking [242,243]. In support of this, recent genome wide association studies identified several endocytosis and trafficking-related genes, such as BIN1, CD2AP, PICALM and CD33, that correlate with the occurrence of AD and are likely to be risk factors [69,244–246].

The majority of endocytic receptors and proteins are trafficked through several well defined intracellular compartments before being delivered to lysosomes for degradation or recycled back towards the membrane. The individual endocytic compartments can be differentiated through members of the rab (ras related protein) GTPase family which play critical roles in controlling vesicular transport between compartments. For instance, rab5 is associated with early endosomes, and in conjunction with the early endosome antigen 1 (EEA1), allows the fusion of endosomes which is required for the formation of other species of endosomes and progression of proteins through the endosomal trafficking pathway [247]. The other main endosomal compartments include the late endosomes, characterised by rab7, which are responsible for movement of proteins to the lysosomal compartments for degradation, and the recycling endosomes, characterised by rab11, which are responsible for the trafficking of proteins back to the cell membrane. While the exact compartments involved in the transcytosis of Aβ across the BBB were not, until recently, very well defined, studies by Zhao et al. have demonstrated that transcytosis of Aβ requires the function of both the early (EEA1 or rab5) and recycling (rab11) endosomes with mutant varieties of each demonstrating significantly reduced Aβ transport across a polarised membrane [248]. In contrast, mutant forms of the late endosome, rab7, resulted in only very modest reductions in Aβ transcytosis.

As previously mentioned, clearance of Aβ through the BBB is believed to be reduced in AD. There are also, there are indications that this process may also be dependent on the apoE isoform present (Chapter 2) [177,179,202]. We propose that one or more of the three processes required for the transcytosis of Aβ in endothelial cells (e.g. endocytosis, endocytic trafficking or exocytosis) is sensitive to the apoE isoform. Indeed, several studies have identified apoE isoform dependent differences in the endocytic trafficking of Aβ. For example, neuronal cells exposed to the apoE3 isoform are more efficient at transporting Aβ to lysosomal compartments compared to apoE4 treated cells [230]. It has also been demonstrated that the apoE2 isoform does not facilitate the uptake of Aβ into neuroblastoma cells in contrast to the apoE3 and apoE4 isoforms [231]. These interactions, in combination, may result in an

increased accumulation of intracellular Aβ with apoE4 compared to the other isoforms [232], potentially as a result of the reduced cellular uptake with apoE2 and increased lysosomal degradation with apoE3. However, the bulk of this work has been conducted in neuronal and microglial cells and not in brain endothelial cells and under the context of transcytosis through the BBB. As such, this chapter will investigate the uptake and intracellular trafficking of Aβ in the presence of different apoE isoforms in HBMECs. This mechanism will be investigated to potentially explain the altered clearance of Aβ through the BBB described in Chapter 2.

3.2 Methods

3.2.1 Antibodies

Immunohistochemical studies were carried out with the following antibodies: rabbit anti-human EEA1 (alexa fluor 647conjugated monoclonal (ab196186, Abcam, USA)), rabbit anti-human rab11 (polyclonal, (ab3612, Abcam, USA)), donkey anti-rabbit IgG (alexa fluor 647 conjugated polyclonal (ab150075 Abcam, USA)), rabbit anti-human LRP1 (alexa fluor350 conjugated polyclonal (bs-5409R-A35, Bioss, USA)).

3.2.2 Preparation of Aβ42 peptides

Aβ42 peptides were prepared in an identical method as previously described in Chapter 2 (section 2.2.3).

3.2.3 Collection and enrichment of human lipidated apoE

Lipidated apoE was collected as previously described in Chapter 2 (section 2.2.4).

3.2.4 ApoE dependent uptake of Aβ42 into HBMECs

Human Brain Microvascular Endothelial Cells (HBMECs) (ScienCell, USA) were seeded into fibronectin-coated 96-well black sided clear bottomed plates and grown in endothelial cell media (ECM) (5% FBS, 5% penicillin/streptomycin, 5% endothelial cell growth serum) (ScienCell). When approximately 90%

confluent, cells were treated with lipidated apoE isoforms (5-50ng/ml) and fluorescein-Aβ42 (2μM) and incubated for 2 hours at 37°C. Following incubation, cells were washed with cold PBS and fixed with 5% PFA. Cell associated Aβ was assessed using a BioTek HT Synergy multidetection microplate reader at 488/515 Ex/Em.

3.2.5 Detection of Intracellular LRP1 in HBMECs after Aβ treatment

HBMECs were seeded into fibronectin-coated 96-well black sided clear bottomed plates and grown in ECM (5% FBS, 5% penicillin/streptomycin, 5% endothelial cell growth serum). When at approximately 90% confluency, cells were treated with lipidated apoE isoforms (25ng/ml) and/or Aβ42 (2μM) before a 2 hour incubation at 37°C. Following incubation, cells were washed with cold PBS and fixed with 5% PFA before permeabilisation of cells with 0.5% Tween 20 for 15 minutes. An anti-LRP1 antibody (1:200) was used to assess the levels of LRP1. The total internalised LRP1 was obtained by subtracting value for the surface LRP1 levels in non-permeabilised cells from the total LRP1 levels in the permeabilised cells. Detection measurements were carried out using a BioTek HT Synergy multidetection microplate reader at 350/440 Ex/Em.

3.2.6 ApoE dependent colocalisation of Aβ with endosomal compartments

HBMECs were seeded onto 8-well chamber slides and grown in ECM 5% FBS, 5% penicillin/streptomycin, 5% endothelial cell growth serum). When approximately 90% confluent, cells were treated with 25ng/ml lipidated apoE. After 24 hours incubation, fluorescein-labelled Aβ42 was spiked in (2μM) and cells were incubated at 37°C for 1 hour. Following incubation, cells were washed with cold PBS and fixed in 100% methanol (EEA1; 5 minutes) or 4% PFA (rab11; 15 minutes) before permeabilisation in 0.3% Triton X-100 for another 5 minutes. Cells were subsequently blocked with 1% FBS, 10% Donkey serum/0.3M glycine and 0.1% PBS/Tween 20 (rab11 only) for 1 hour before incubation with primary antibodies overnight at 4°C (EEA1 1:50, rab11 1:200), . When necessary, cells were washed and incubated with the secondary antibody (alexa fluor 647 conjugated donkey anti-rabbit IgG, 1:200) for 2

hours at room temperature. Otherwise, all slides were washed with PBS before mounting with Fluoroshield mounting media with DAPI (Sigma Aldrich, USA). All were single images and were taken with a Zeiss LSM 800 confocal microscope (Zeiss, Germany). For imaging and quantification analysis, 5 randomly selected images were used from each biological replicate. Colocalisation coefficients, endosome size, and area coverage were assessed using Zen blue edition 2.1 software (2011) (Zeiss, Germany).

3.2.7 Statistical analysis

Where appropriate, significance values were obtained through one-way or two-way ANOVA followed by Tukey *post-hoc* analysis (GraphPad Prism 5, GraphPad Software Inc., USA). Otherwise, where only 2 groups were compared, unpaired t-tests were utilised. Unless otherwise indicated, all n values represent technical replicates.

3.3 Results

3.3.1 ApoE dependent uptake of Aβ

Uptake of Aβ into cultured endothelial cells was found to be apoE dependent, with apoE3 and apoE4 displaying the highest levels of cell associated Aβ (cell surface and internalised) after a 2 hour treatment (Figure 3.1). The lowest levels of cell associated Aβ in was detected in cells treated with 50ng/ml apoE2 and was approximately 40% lower than the untreated controls and was significantly lower than the apoE4 treated cells.

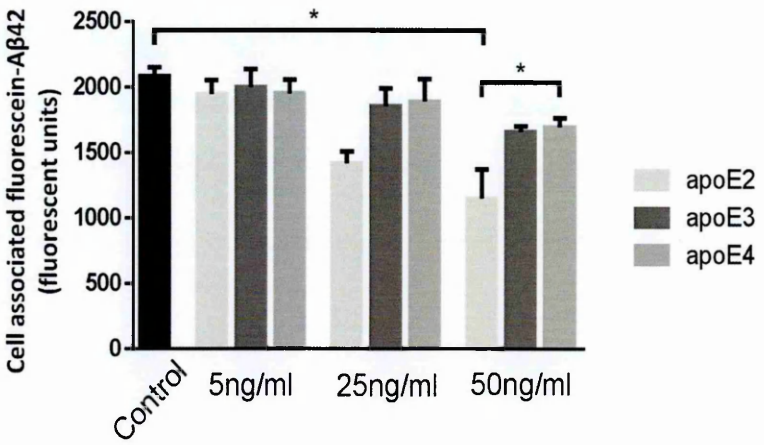


Figure 3.1 : Uptake of fluorescein-Aβ42 in HBMECs with different apoE isoforms. HBMECs were exposed to apoE isoforms (5-50ng/ml) and fluorescein-Aβ42 (2μM) for 2 hours and the level of cell associated fluorescein-Aβ42 measured. Cells exposed to apoE4 demonstrated a significantly higher levels of cell associated fluorescein-Aβ42 when compared to apoE2 treated cells at 50ng/ml apoE. In addition, apoE2 treated cells showed a reduction of cell associated fluorescein-Aβ42 when compared to the untreated control. Values represent mean ± SEM (n = 3) and are expressed as mean fluorescent units. *p<0.05 as determined by one-way ANOVA followed by Tukey's *post-hoc* analysis.

3.3.2 The level of intracellular LRP1 is apoE isoform dependent

The levels of intracellular LRP1 after exposure to Aβ was found to be apoE isoform dependent (apoE2<apoE3<apoE4) (Figure 3.2). Levels of intracellular LRP1 in the apoE only cells were consistently lower than in the cells exposed to both apoE and Aβ. However, only apoE3 and apoE4 exposed cells reached statistical significance. In the control cells (no apoE exposure), treatment with Aβ did not appreciably alter the levels of intracellular LRP1, and was the only treatment group that showed a reduction, albeit only modestly, in the levels of intracellular LRP1. However, the untreated controls (no

Aβ treatment or apoE) showed significantly higher levels of intracellular LRP1 compared to all of the apoE treated cells.

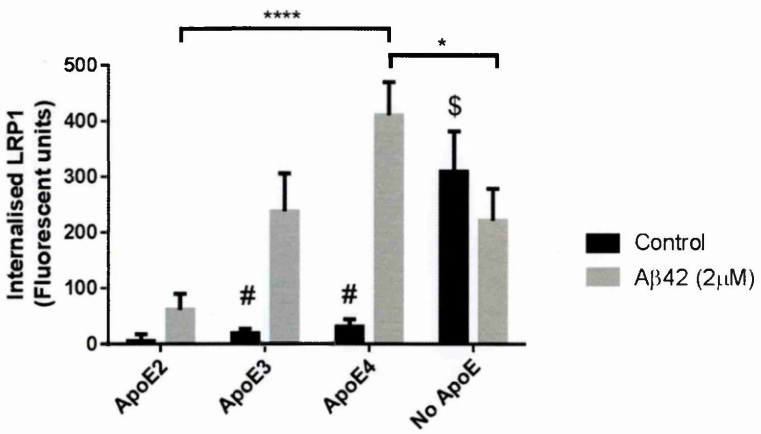


Figure 3.2 : Levels of intracellular LRP1 after exposure of HBMECs to apoE and Aβ42. ApoE isoform dependent levels of intracellular LRP1 was observed with apoE4 demonstrating the highest levels of internalised LRP1 after exposure to Aβ42. Treatment induced significant increases in the amount of intracellular LRP1 in both the apoE3 and apoE4 treated cells compared to the respective control. In addition, cells with no apoE showed higher levels of intracellular LRP1 compared to all apoE isoforms in the absence of Aβ42. Values represent mean ± SEM (n = 8) and are expressed as mean fluorescent units. *p<0.05, ****p<0.0001, $ p< 0.05 compared to all other conditions in the treatment group, #p<0.01 between treatment and respective control. Statistics determined by two-way ANOVA followed by Sidak *post-hoc* analysis.

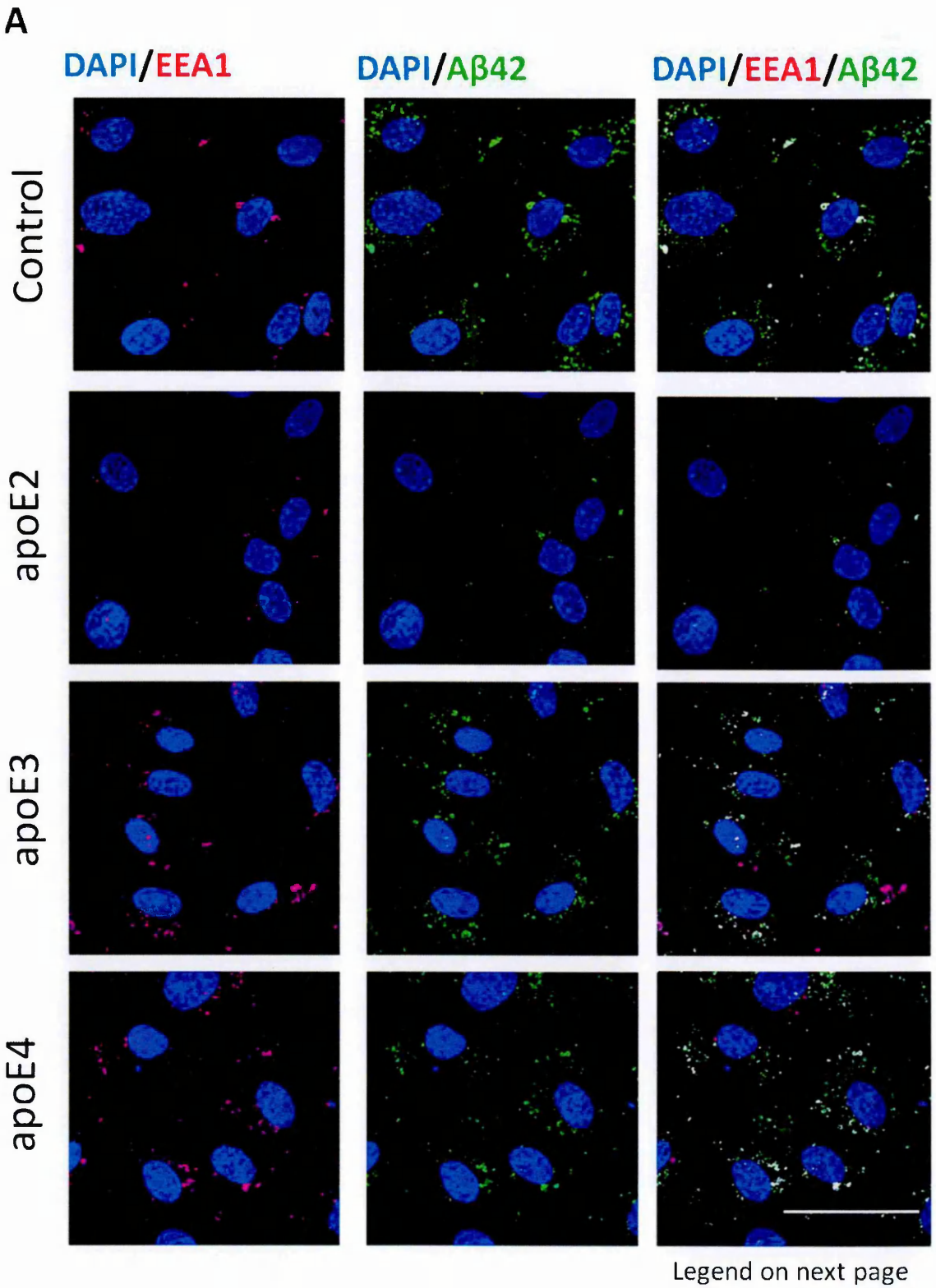
3.3.3 Colocalisation of Aβ with EEA1 and rab11 endosomal markers

Areas that stained positive with the EEA1 marker showed high levels of association with Aβ. In addition, this interaction was found to be apoE dependent, with EEA1 positive areas and Aβ colocalisation the lowest in apoE2 treated cells and highest in the untreated controls (apoE2<apoE3<apoE4<control) (Figure 3.3 A and B). The fraction of the total cell associated Aβ that was localised with EEA1 positive

areas (Aβ-EEA1 colocalisation coefficient) was lowest in the control and apoE2 treated cells and highest in the apoE4 treated cells (apoE2<control<apoE3<apoE4) (Figure 3.3 A and C). In contrast, no significant differences in the colocalisation of the recycling endosomal marker, rab11, with Aβ were observed (rab11-Aβ colocalisation coefficient). Similarly, the amount of Aβ that was associated with the areas positive for recycling endosomes was not affected by the apoE isoform present (Aβ-rab11 colocalisation coefficient) (Figure 3.4 A-C).

3.3.4 Quantification of EEA1 and rab11 endosome number and size

ApoE dependent effects on the both the number and size of the EEA1 positive early endosomes were observed (Figure 3.3 A, D and E). ApoE2 treated cells showed the lowest number of EEA1 early endosomes (control=apoE2<apoE3<apoE4) in addition to the endosomes being significantly smaller than those in the apoE3 and apoE4 treated cells (control=apoE2<apoE3=apoE4). In comparison, no significant differences in the size or number of rab11 positive recycling endosomes were detected between the any of the apoE treatments (Figure 3.4 A, C and D).



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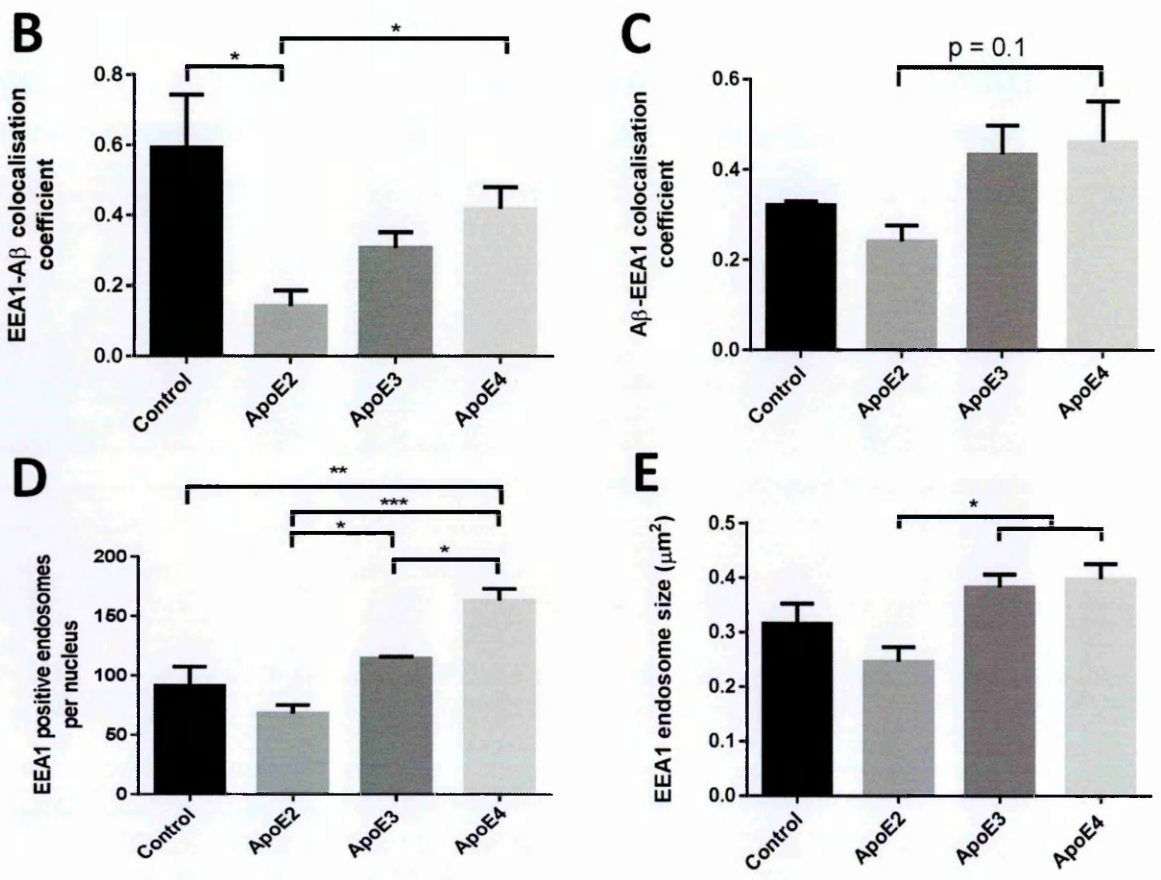


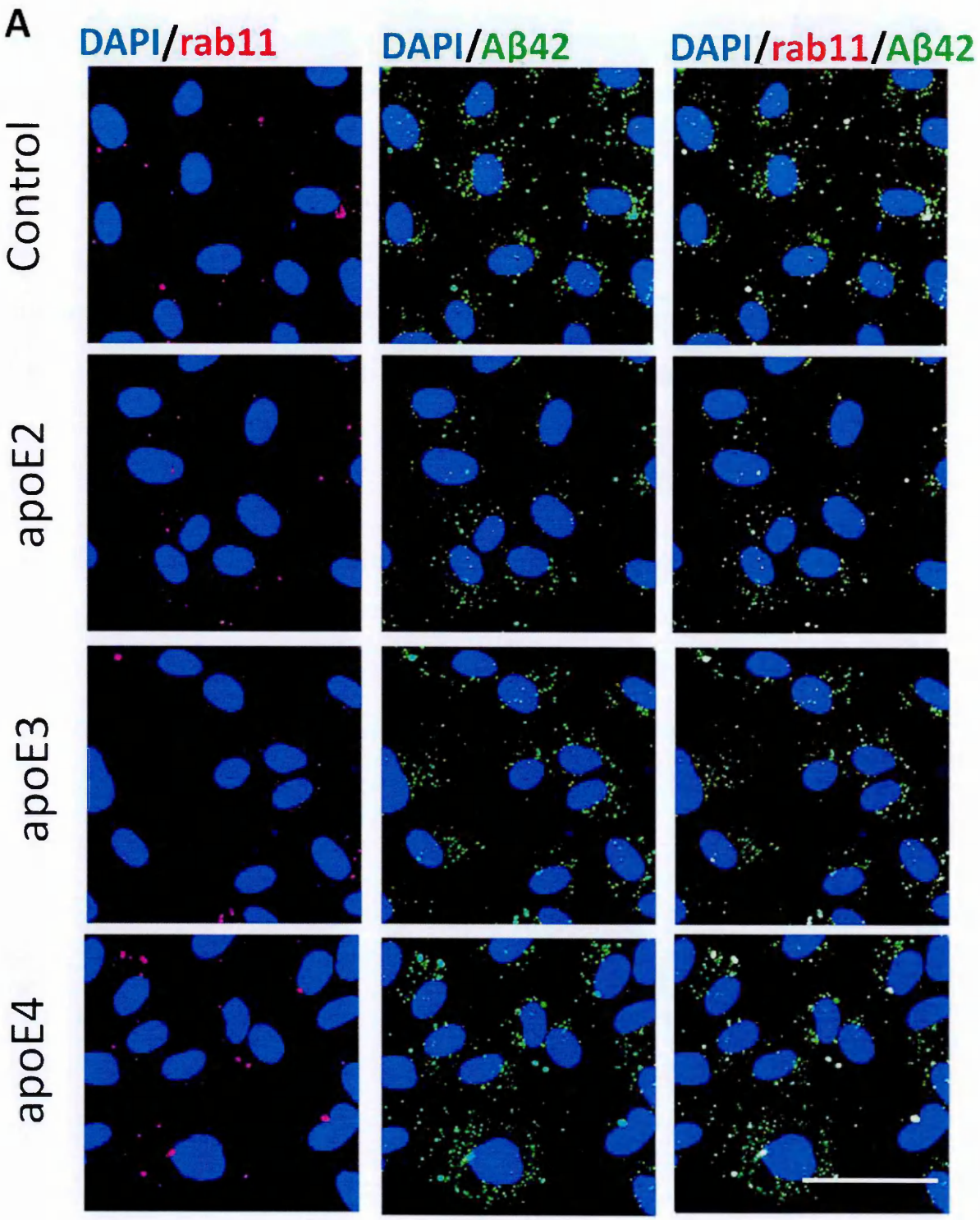
Figure 3.3 : ApoE isoform-dependent impact on EEA1 positive early endosomes and Aβ42 trafficking. (A) HBMECs were exposed to apoE (25ng/ml) for 24 hours before treatment with fluorescein-Aβ42 (2μM) for 1 hour. (B) The fraction of EEA1 positive early endosomes that contained Aβ42 was apoE dependent with the control and apoE4 isoform treat cells demonstrating the highest EEA1-Aβ42 colocalisation. (C) In addition, there was an increase in the fraction of total cell associated Aβ42 that was colocalised with EEA1 in the apoE4 isoforms when compared to apoE2, apoE3 and the control cells. (D) The number of EEA1 positive early endosomes was higher in cells in an apoE4 environment compared to all other apoE isoforms and the control. (E) In addition, early endosomes were significantly larger in the apoE3 and apoE4 treated cells in comparison to the apoE2 treated cells. Scale bar (located on DAPI/EEA1/Aβ42 apoE4 treated panel) represents 50μm. Values represent mean

± SEM (n = 5 technical replicates from each of the 3 biological replicates). *p<0.05, **p<0.01, ***p<0.001 as determined by one-way ANOVA followed by Tukey's *post-hoc* analysis.

3.4 Discussion

The accumulation of Aβ that is found in the AD brain may be the result of reduced or dysfunctional Aβ clearance [75]. One of the major routes of clearance of Aβ from the brain is through the BBB with at least 25% of total clearance reliant on this route [101]. This process requires the initial uptake of Aβ into the cell, via receptors such as LRP1, and endocytic trafficking leading to the eventual release of the cargo at the apical side of the BBB into the plasma.

As previously discussed, the presence of the APOE4 allele is a major risk factor for the development of AD. Multiple studies have shown an association between APOE4 and Aβ levels in the brain and as such, is strongly implicated in the development AD pathology and the onset of disease. In contrast, the APOE2 allele is associated with a lowered risk of developing AD in comparison to the APOE3 allele and appears to confer a protective effect with regards to Aβ pathology. We initially investigated whether the internalisation Aβ in endothelial cells was altered by the presence of the different apoE isoforms. Interestingly, we found that apoE2 significantly reduced the amount of Aβ associated with the cells. In addition, this was supported by experiments which showed reduced internalisation of LRP1 in HBMECs after treatment with Aβ42 in cells exposed to apoE2 when compared to the other apoE isoforms. While this at first appears to contradict the transcytosis data outlined in Chapter 2, with apoE2 resulting in the highest levels of transcytosis and apoE4 the lowest, closer inspection of the subcellular localisation of Aβ suggests that subcellular trafficking may be a confounding factor that could help explain this apparent contradiction. It is also possible that the reduced internalisation of Aβ and LRP1 in the apoE2 treated cells could also be attributed to an increase in the rate of receptor or ligand recycling. Under this scenario the rate of internalisation would be more closely matched by the rate of exocytosis in the



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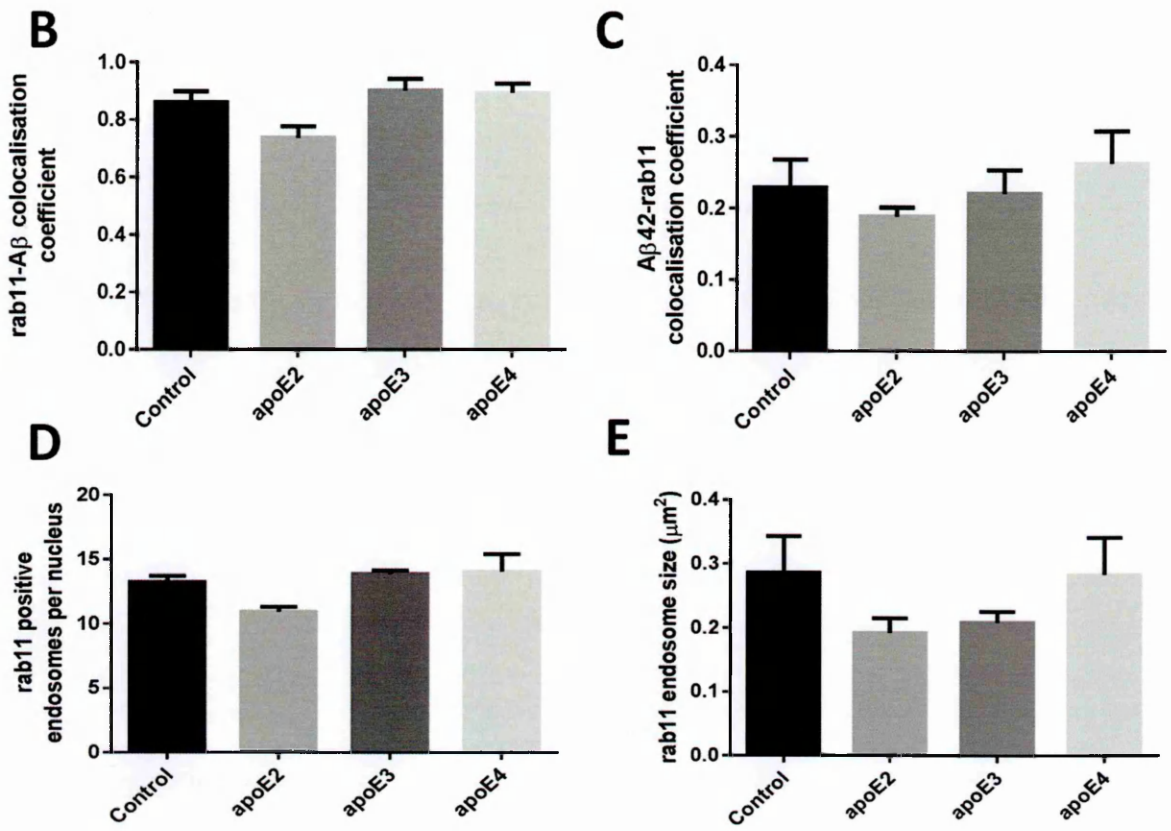

Figure 3.4 ApoE-isoform dependent impact on rab11 positive recycling endosomes and Aβ42 trafficking. (A) HBMECs were exposed to the apoE isoforms (25ng/ml) for 24 hours before treatment with fluorescein-Aβ42 (2μM) for 1 hour. (B) Treatment of cells with apoE did not significantly alter the number of recycling endosomes that contain Aβ42 or (C) the fraction of total cell associated Aβ42 that was colocalised with the rab11 positive recycling endosomes. In addition, neither (C) the number or (D) size of the rab11 positive endosomes was significantly altered by the presence of the different apoE isoforms. Scale bar (located on DAPI/rab11/Aβ42 apoE4 treated panel) represents 50μm. Values represent mean ± SEM (n = 5 technical replicates from each of the 3 biological replicates) and statistical significance assessed via one-way ANOVA.

apoE2 cells. Conversely, under apoE4 conditions, the rate of recycling may be slower, leading to an intracellular accumulation of Aβ. However, under this experimental paradigm, it is impossible to conclusively state which of these two explanations is correct.

It has previously been demonstrated that both early and recycling endosomal compartments are required for the efficient transcytosis of Aβ across the BBB [248]. In AD, both neurons and fibroblasts show early endosome dysfunction which is characterised by both an increase in number and size of early endosomes [249–251]. Furthermore, increases in the expression of early and late endosomal markers were detected in the hippocampus of brains from patients with mild-cognitive impairment (MCI) and AD [252]. This increase in endosomal markers was found to correlate with cognitive decline, suggesting it may be an early pathological event in the development of AD [235]. In our study, we observed an increase in the number of early endosome positive areas in endothelial cells in the apoE3 and apoE4 treated cells compared to the apoE2 cells. This suggests that the pathologies associated with increased early endosomal accumulation, which based on the available evidence appears to be fairly ubiquitous between cell types, may be influenced by the apoE isoform that is present. In addition, an increase in the size of the early endosome positive area was also detected in the apoE3, apoE4 and control (no apoE) endothelial cells compared to the apoE2.

Although not previously identified in endothelial cells, the role of dysfunctional endosomal transport in the early stages of AD has been highlighted in studies carried out by Cataldo et al. who demonstrated that differences in the size of endosomes in neurons. In addition, these endosomal abnormalities were accelerated by the apoE4 allele at the earliest stages of disease, but not in moderate to severe stages [253]. Furthermore, knockdown of proteins involved in the control of the late endosomes in neuronal like cells prevented trafficking of Aβ to the lysosomal compartments, resulting in enlargement of the early endosomes and accumulation of Aβ in the early endosomes, similar to that witnessed in our study

[254]. This suggests that the enlarged early endosomes may occur as a result of an inability to efficiently traffic protein cargo to the next appropriate endosomal compartment. The enlarged endosomes in our study are colocalised with Aβ, suggesting that Aβ peptides may be accumulating in these swollen early endosomes. If this change in number and morphology of early endosomes is revealed to be a pathological driver, it suggests that the both the absence of any apoE isoform, or the presence of the apoE3 and apoE4 isoforms may be detrimental to endosomal trafficking of Aβ in the cerebrovasculature and would therefore impact the BBB clearance of Aβ. In contrast, it has previously been suggested that the increase in number and size of early endosomes may be a compensatory mechanism that occurs due to the elevated levels of Aβ found in the AD brain [250]. However, our study suggests that this is not the case, as all cells received the same amount of Aβ and the pathology was only reproduced in the control, apoE3 and apoE4 treated cells.

In addition, other abnormalities in the composition of the early endosomes have also been noted in AD. In particular, high levels of the proteases Cathepsin B and D, which are normally concentrated in the lysosomal compartments, are found in the early endosomes of neurons in regions with increased levels of Aβ [255,256]. While it has not yet been established whether the change in localisation of these proteases is a driver of pathology, or simply a reaction to increased endocytosis of Aβ in AD, there are indications that Cathepsin is detrimental to memory tasks in AD animal models. For instance, Cathepsin deletion in an AD mouse model significantly improved learning and memory retention while also reducing the brain Aβ load. Therefore, the increased expression of Cathepsin may be a contributor to AD pathology [257].

The efficient transcytosis of Aβ also relies on the recycling endosomes, characterised by the expression of rab11 [248]. While the majority of research in the AD field has focused on the dysfunction of early and late endosomes, there are also indications that the activity of the recycling endosomes may also be impaired. For instance, the expression of rab4, a marker for rapid recycling endosomes, is upregulated in

MCI and in AD [235]. This upregulation of rab4 was observed in the MCI cohort when compared to cognitively normal controls. In addition, no change in rab4 expression detected between the MCI and AD groups, suggesting that this may be one of the early pathologies associated with the development of MCI prior to the conversion to AD [235]. However, in our study, we observed no significant difference in the size or morphology of the rab11 positive recycling endosomes. In addition, there was also no apoE dependent effect on the colocalisation of Aβ with rab11 or the number of rab11 endosomes that contained Aβ. However, while the rab11 recycling endosomes appear unaffected by the apoE isoforms preset, it is possible that the rapid recycling endosomal compartments which were found to be altered by MCI and AD, as characterised by the rab5 protein, are sensitive to the apoE isoforms which may explain some risk conferred by the APOE4 gene [235].

In summary, evidence from these studies suggest that the apoE4 isoform causes an increase in the levels of internalised Aβ which does not correspond with an increase in the transcytosis of Aβ across the BBB, actually resulting in the slowest clearance across the BBB (as outlined in Chapter 2). Therefore, the rate in which Aβ is shuttled through the endosomal compartments after its initial internalisation must be slower in the apoE4 environment compared to the apoE2. This may help explain the observation for reduced internalisation of Aβ in an apoE2 environment as the recycling may be quicker, resulting in a reduced backlog of Aβ. We hypothesise that the enlarged early endosomes detected with the apoE4 isoform, which have also been observed in the AD brain, may be the result of this slower trafficking of Aβ between the early and the recycling endosomes [253]. However, due to the design of this study, with only a final endpoint assessment of the intracellular trafficking, we were not able to assess any changes in the rate of Aβ transport. Regardless, it is clear that while more internalised Aβ is associated with the presence of apoE4 or in the absence of any apoE isoform, more Aβ appears to accumulate in the early endosomes, which potentially explains the diminished elimination from the endothelial cells and transcytosis described in Chapter 2 (Figure 2.5). However, in contrast to the previous studies investigating the clearance of Aβ across the BBB *in vitro* [179,216], the polarity of the cells in the

subcellular localisation studies was not assessed. Therefore, it is feasible that changes in directional transport and therefore endosomal trafficking may be altered under non-polarised conditions. Regardless, changes in internalisation and the early stages of endosomal trafficking were detected which may not be significantly influenced by polarisation.

Although these studies provide mechanistic insight into the influence of the apoE4 isoform on Aβ transit through the BBB, the endosomal trafficking of Aβ may be challenging to target pharmacologically. To our knowledge, no current therapeutics directly alter the trafficking of proteins through the endosomal compartments. Therefore, additional strategies may be required to facilitate the uptake of Aβ and its subsequent clearance across the BBB. The following chapters will investigate the enzymes responsible for the ectodomain shedding of LRP1 (Chapter 2) as a potential therapeutic target to facilitate Aβ elimination from the brain and mitigate the impact of APOE4 on the AD phenotype.

Chapter 4 : The influence of ADAM10 on LRP1 shedding and clearance of Aβ across the BBB

4.1 Introduction

As outlined in Chapter 2, the ectodomain shedding of LRP1 and the formation of a soluble LRP1 specifies inversely correlates with the clearance of Aβ BBB [202]. Thus, reducing brain LRP1 shedding could promote Aβ clearance across the BBB and attenuate Aβ accumulation in the AD brain. As such, investigating the factors that regulate LRP1 shedding in the brain may provide therapeutic opportunities to lower Aβ burden and modulate the AD phenotype. One of the enzymes implicated in LRP1 ectodomain shedding is the α-secretase, ADAM10 (A Disintegrin And Metalloproteinase domain containing protein 10) [146]. The following study will examine the influence of ADAM10 on LRP1 shedding *in vitro* and *in vivo* and evaluate the impact of ADAM10 modulation on Aβ clearance across the BBB.

The majority of research investigating the involvement of ADAM10 on the pathological mechanisms that occur in AD have focused on its function as an α-secretase. In this role, ADAM10 proteolytic activity results in the formation of sAPPα from the amyloid precursor protein (APP) which has been reported to exhibit neuroprotective and neurotropic properties (Figure 1.1) [258,259]. Perhaps more importantly, ADAM10 also competes with the β-secretase base for cleavage of APP such that an increase in ADAM10 activity reduces the generation of Aβ through the β-secretase mediated amyloidogenic processing of APP [260]. The importance of ADAM10 function with regards to APP processing was highlighted by the discovery of two rare highly penetrant mutations (Q170H and R181G) that were identified in the ADAM10 prodomain that resulted in reduced α-secretase activity [261]. When introduced into models of AD over-expressing APP, there was an increase in Aβ production in cell culture and an increase the brain Aβ burden in mice [261,262]. However, ADAM10 has a diverse array of substrates that are

involved in the regulation of vascular development and inflammatory [263]. As such, increased ADAM10 activity is associated with several disease pathologies including atherosclerotic cerebral infarction, restenosis and in early tumourigenesis events [264–266]. ADAM10 is also shows proteolytic activity against several proteins that are integral for the proper function of the BBB [267,268]. This endothelial and tight junction specific activity of ADAM10 suggests that it may be involved in the well documented vascular dysfunction that is a common feature of AD [269]. In addition, recent findings linking ADAM10 activity to the ectodomain shedding of LRP1 further supports this evidence that ADAM10 may be involved in the BBB pathologies associated with AD and the clearance of Aβ through the BBB.

As previously discussed, APOE4 constitutes the strongest genetic risk factor for AD, with possession of one or two APOE4 alleles increasing the chance of developing AD by 4- and 15- fold, respectively, compared to APOE3 homozygous individuals [160,162]. However, few studies have assessed the potential interaction between apoE and the activity of ADAM10. Of the studies that have assessed this interaction, they primarily focus on the non-amyloidogenic processing of amyloid-precursor protein (APP) [270]. The Aβ induced shedding of LRP1 outlined in Chapter 2 was also found to be apoE dependent, indicating that the activity of the protease responsible for the shedding of LRP1 may also be influenced by apoE genotype. Therefore, in addition to examining the effect of ADAM10 on LRP1 shedding, the following studies will also evaluate the interaction between ADAM10 and each apoE isoforms.

4.2 Methods

4.2.1 Animals

ADAM10 endothelial KO mice were kindly provided by Dr Carl Blobel (Hospital for Special Surgery, New York, NY), Dr B. De Strooper (VIB Centre for the biology of Disease, Leuven, Belgium) and Dr P. Saftig

(Institute of Biochemistry, CAU Kiel, Germany). These mice were generated by crossing ADAM10 flox/flox mice [271] with Tie2-Cre transgenic mice that use the endothelial specific promoter Tie2 to drive the expression of Cre recombinase [272], which result in ADAM10 flox/flox/Tie2-Cre mice. The ADAM10 endothelial KO mice lack ADAM10 expression in the endothelial cells [273]. Wild Type mice on a C57BL/6 background were used as control animals. To evaluate the effect of ADAM10 modulation on BBB clearance of Aβ in an animal model of AD, we utilized transgenic mice overexpressing the human APP695 sw mutation and the presenilin-1 mutation M1 46L (PSAPP) which results in the overproduction of human Aβ [274]. All mice were group housed in a temperature and humidity controlled environment on a 12 hour light/dark cycle with free access to food and water. All experiments involving animals were approved by the Institutional Animal Care and Use Committee of the Roskamp Institute.

4.2.2 Preparation of Aβ42 peptides

Aβ42 peptides were prepared in an identical method as previously described in Chapter 2 (section 2.2.3).

4.2.3 Influence of ADAM10 on transit of Aβ across an *in vitro* model of the BBB

The impact of ADAM10 on Aβ42 transcytosis was assessed using an *in vitro* BBB model previously described by our group [216]. HBMECs were seeded onto fibronectin-coated 24-well membrane inserts forming a polarised monolayer representing the BBB (Figure 4.1). Aβ transcytosis across the BBB was assessed by exposing the basolateral side ("brain") to 2μM fluorescein-Aβ42 and treating the apical side ("blood") with the ADAM10-selective inhibitor GI254023X (0.1-1μM) (stock solution in DMSO). The concentration of DMSO in treatment wells was <0.1%. Previous studies have demonstrated that DMSO at these concentrations does not affect the permeability in this BBB model and the conjugation of Aβ42 to fluorescein does not significantly alter the rate of transcytosis of unconjugated Aβ42 through the polarised membrane [216]. After 60 minutes of incubation at 37°C, samples were collected from the apical compartment to assess the basolateral-to-apical transcytosis of fluorescein-Aβ across the BBB. To

ensure the integrity of the barrier was intact, the basolateral-to-apical permeability of a paracellular marker, 10kDa lucifer yellow dextran, across the BBB model was examined for each study. The levels of fluorescein-Aβ in the apical compartment were assessed via fluorescence at 485/515 Ex/Em using BioTek Synergy HT microplate reader.

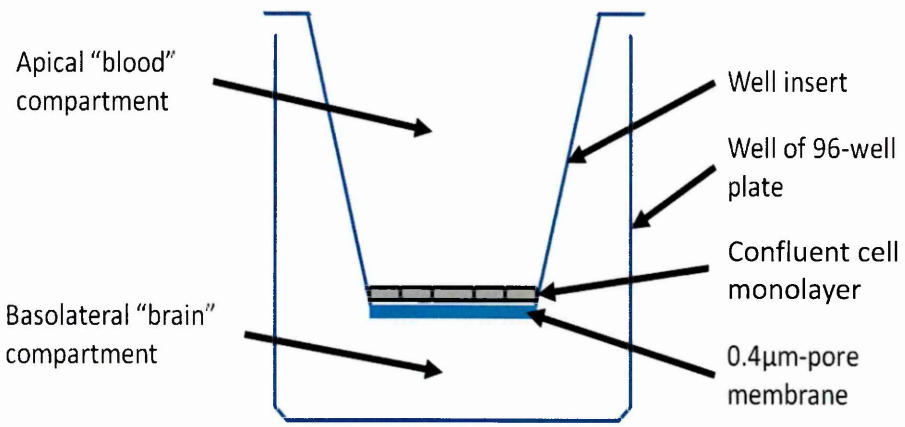

Figure 4.1 Schematic of *in vitro* BBB model well inserts

4.2.4 Brain LRP1 shedding and Aβ BBB clearance *in vivo*

To determine the role of ADAM10 in the clearance of Aβ42 from the brain to the periphery, we examined the appearance of human Aβ42 in the plasma after intracranial administration of human Aβ42 as previously described by our group [216,221]. Briefly, wild-type and ADAM10 endothelial KO mice (4 to 6 months of age) were anesthetized via inhalation using a 3% isoflurane / oxygen mix. While under anaesthesia, vehicle (100% DMSO) or 3µl of human Aβ42 (1mM) was bilaterally injected into the caudate putamen of the brain (0.5mm anterior to the bregma and 2mm lateral to the midline at a depth of 3mm below the skull surface) with approximately 1 minute between the bilateral injections. Ten

minutes after the intracerebral injections, the mice were euthanized and the plasma and brain tissue were collected. Plasma samples were analyzed for human Aβ42 using an ELISA (Invitrogen Corp., USA). Mouse brains were homogenised in 12ml of ice cold Hanks Balances Salt Solution (HBSS) with a Dounce Homogenizer. For collection of the soluble brain fraction (i.e., non cell-associated), samples were centrifuged at 6000g for 15 minutes to remove cellular debris and non-soluble components. The levels of LRP1 in the soluble fraction were measured using a human LRP1 ELISA (Cedar Lane Labs, USA) and normalized to the total protein content in the brain homogenate as determined by the bicinchoninic acid (BCA) protein assay (Thermo Scientific, USA). Soluble LRP1 levels were expressed as ng of LRP1 per mg protein.

4.2.5 Aβ and sAPPα levels in PSAPP mice following ADAM10 inhibition

PSAPP mice at 35 weeks of age were injected intraperitoneally with 200mg/kg of GI254023X or vehicle (DMSO) once per day for 5 consecutive days. At this age, PSAPP mice display elevated levels of Aβ peptides in the brain [274]. This treatment regime has previously been shown to effectively inhibit ADAM10 activity in mice and was well tolerated with no adverse effects reported [275]. One hour after the final injection, the mice were euthanatized and plasma and brain tissue was collected. The right hemisphere was used to examine the levels of soluble and insoluble Aβ40 and Aβ42 in the brain. Briefly, the hemispheres were homogenized by sonication in 700µl lysis buffer (M-PER + 1%EDTA +0.2%PMSF (Thermo Scientific, USA)) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, USA) on ice before centrifugation at 14000g for 30 minutes at 4°C. 100µl of the resulting supernatant was mixed with 5M guanidine in TRIS buffer resulting in the guanidine soluble (GS) fraction. For the guanidine insoluble (GI) fraction, 100µl of the guanidine stock was combined with the original tissue pellet. Both GS and GI fractions were subsequently incubated at room temperature for 1 hour and were mixed every 15 minutes. All samples were stored at -80°C prior to analysis. Quantification of Aβ40 and Aβ42 in the GS, GI, and plasma fractions was carried out using an ELISA for human Aβ40 and Aβ42 (Invitrogen, USA) and expressed as percentage of vehicle control. The left hemisphere was used to

examine the levels of soluble LRP1 in the brain. Here, the hemisphere was homogenized using a Dounce homogenizer in ice cold in HBSS using the same procedure as the *in vivo* LRP1 shedding studies above. LRP1 levels in the soluble fraction were assessed by ELISA for mouse LRP1 (Cedar Lane Labs, USA) and normalized to the total protein content in the brain homogenate as determined by the BCA protein assay (Thermo Scientific, USA). Soluble LRP1 levels were expressed as ng of LRP1 per mg protein. In addition, sAPPα levels were examined in the brain homogenate of these same animals using an ELISA for sAPPα (IBL international, USA). The sAPPα levels were normalized to the total protein content in the brain homogenate as determined by the BCA protein assay and expressed as ng of sAPPα per mg protein.

4.2.6 *In silico* BBB permeability of GI254023X

Values for the prediction of the permeability of GI254023X through the BBB were calculated using Molinspiration online resources (www.molinspiration.com). Values obtained were the Octanol-water partition coefficient logP (miLogP), topical polar surface area (TPSA in A^2), Molecular weight (MW), and sum of Oxygen and Nitrogen atoms. Common values in which a compound is considered permeable through the BBB are as follows: miLogP - Oxygen and Nitrogen total > 0, TPSA<90 [276] or <60-70 A^2 [277], MW< 400Da [187] to 450Da [278] , and the Oxygen and Nitrogen total < 5 [279]. These values were used to assess the likelihood of permeability utilising 4 of the rules of thumb outlined by Clarke [30]. Additional BBB permeability prediction tests were conducted using resources on www.cbligands.org with a SVM algorithm and 4 separate molecular fingerprints. A positive control compound, anatabine, which has previously been shown by our group to cross the BBB was assessed using the same method to confirm the validity of the tests [221].

4.2.7 Collection and enrichment of human lipidated apoE

Lipidated apoE was collected as previously described in Chapter 2.2.4.

4.2.8 ApoE-dependent ADAM10 activity

To assess whether any of the ADAM10 dependent effects are modulated by apoE, we assessed the activity of ADAM10 in the presence of the apoE isoforms in a cell-free paradigm. Recombinant ADAM10 (1μM) (R&D Systems, Canada) was incubated with lipidated apoE2, apoE3 and apoE4 (0.5-250ng/ml) and the fluorescent substrate PEPDAB010 (5nM) (BioZyme Inc., USA) for 1 hour at 37°C. Additionally, the kinetic activity of ADAM10 (1μM) was carried out in the presence of apoE (0.1 - 300ng/ml) and a fixed concentration of PEPDAB010 (5nM). In this study, ADAM10 and apoE were preincubated at 37°C for 1 hour before addition of PEPDAB010. Enzymatic activity was then measured by fluorescence every 35 seconds for 30 minutes at 37°C. The rate of reaction for each concentration of apoE was calculated by taking the initial gradient of the reaction rate in the linear region (fluorescent units/seconds) and plotting it against the concentration of apoE. In both the activity and kinetics cell-free assays, to determine whether the substrate was metabolised by apoE itself, fluorescent substrate was incubated with various concentrations of apoE alone. These values were used as background controls for each apoE isoform-ADAM10 treatment combination. For all cell-free activity paradigms, fluorescence was measured at 485/528 Ex/Em using a BioTek HT Synergy multidetection microplate reader.

4.2.9 Statistical analysis

Statistical analyses were performed using a one-way ANOVA followed by Tukey's *post-hoc* analysis (GraphPad Prism 5, GraphPad Software Inc., USA). Moreover, where only 2 groups were compared, unpaired t-tests were used to evaluate statistical significance. Km values were generated using a nonlinear dose-response regression of the data set (Michaelis Menten curve fit). Unless otherwise indicated, all n values represent technical replicates.

4.3 Results

4.3.1 Influence of ADAM10 on transit of Aβ across an *in vitro* BBB model

Previously, we demonstrated that inhibition of ADAM10 with GI254023X reduced the ectodomain shedding of LRP1 (Figure 2.7). Leading on from these findings, we assessed whether ADAM10 inhibition, and therefore the reduction LRP1 shedding, could increase the transcytosis of fluorescein-Aβ42 across in an *in vitro* BBB model. Treatment of HBMECs with GI254023X at concentrations increased the basolateral to apical transport of fluorescein-Aβ42 in a dose dependent manner (Figure 4.2). Significant increases were observed at concentrations higher than 1μM GI254023X showing effects of 1.25-fold and greater. In addition, treatment did not significantly increase the amount of 10kDa lucifer yellow dextran in the apical compartment, suggesting the integrity of the confluent cell monolayer was not impacted by treatment.

4.3.2 Brain LRP1 shedding and Aβ BBB clearance *in vivo*

LRP1 levels in the soluble brain fraction of ADAM10 endothelial KO mice were lower than that observed in wild-type animals (

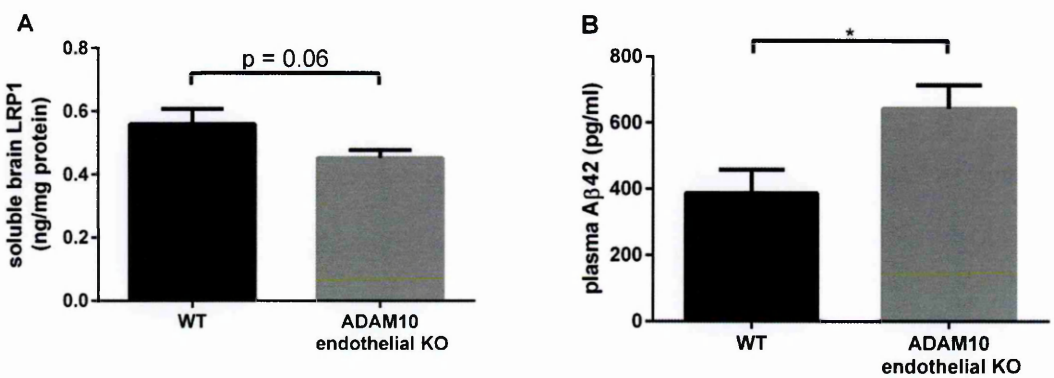

Figure 4.3), although this effect did not achieve statistical significance. However, Aβ clearance across the BBB was significantly greater in ADAM10 endothelial KO mice compared to wild-type animals resulting in an increase of approximately 1.75-fold.

4.3.3 ADAM10 inhibition in PSAPP mice

To evaluate the impact of ADAM10 inhibition on Aβ tissue levels and LRP1 shedding in an AD animal model, PSAPP mice were treated with the ADAM10-selective inhibitor GI254023X. The level of soluble LRP1 in the brain was significantly lower in the GI254023X-treated mice compared to the vehicle control animals (a reduction of 60%) (Figure 4.4 A). To assess the effect of ADAM10 inhibition on the α-secretase cleavage of APP, the levels of sAPPα in the brain were examined following GI254023X treatment. No significant difference in the level of sAPPα in the brain was detected between GI254023X-treated mice and the control group (Figure 4.4 B). The involvement of ADAM10 in the clearance of Aβ was assessed by measurement of Aβ40 and Aβ42 in the plasma and whole brain homogenate.

ADAM10 inhibition significantly increased the levels of Aβ40 in the plasma (1.45-fold) compared to vehicle-treated mice, while the effect of GI254023X treatment on plasma Aβ42 showed no significant change (Figure 4.5A). In addition, treatment with the ADAM10 inhibitor reduced both soluble and insoluble Aβ40 (Figure 4.5B) (1.15- and 1.20-fold respectively) and Aβ42 (Figure 4.5C) (1.20 and 1.25-fold respectively) levels in the brain compared to vehicle-treated animals, although these values did not reach statistical significance. It should also be noted that this GI254023X treatment regimen was well tolerated as the treated animals did not display any overt changes in appearance, behaviour, or weight loss.

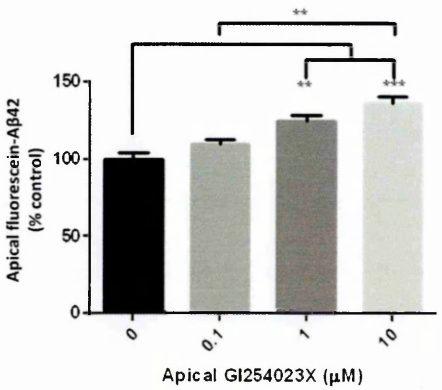

Figure 4.2: Fluorescein-Aβ42 transit across an *in vitro* BBB model following ADAM10 inhibition. Fluorescein-Aβ42 (2μM) was exposed to the basolateral ("brain") side of the *in vitro* BBB model, while various concentrations of the ADAM10 inhibitor GI254023X (0-10μM) were exposed to the apical ("blood") compartment. Following incubation at 37°C, samples were collected from the basolateral compartment at 60 minutes to assess the basolateral-to-apical transcytosis of fluorescein-Aβ42 across the BBB model. Values represent mean ± SEM (n = 3) and are expressed as the percentage change from control conditions. *p<0.05; ** p<0.01; *** p<0.001 as determined by one-way ANOVA followed by Tukey's *post-hoc* analysis.

4.3.4 *In silico* BBB permeability of GI254023X

Prediction of BBB permeability of GI254023X was based on the values for the miLogP, TPSA, MW and the sum of Oxygen and Nitrogen atoms. The values obtained for GI254023X were as follows: MW = 391.51Da (BBB permeable), TPSA = 98.73 A^2 (BBB impermeable), sum of Nitrogen and Oxygen = 7 (BBB impermeable), miLogP - (N+O) = -4.4 (BBB impermeable). The additional BBB permeability prediction software (cbligands) predicted that GI254023X would be permeable in 3 out of the 4 molecular fingerprints. Overall, this suggests that GI254023X may have marginal permeability through the BBB. Our positive control, anatabine which has been previously been demonstrated to penetrate the BBB was predicted to penetrate the BBB in all of the tests performed [280].

4.3.5 Cell-free apoE isoform dependent ADAM10 activity

To determine the effect of the apoE isoforms on the activity of ADAM10, a cell-free activity assay was utilised (Figure 4.6A). Following exposure of apoE isoforms and an ADAM10 fluorescent substrate to active recombinant ADAM10, apoE isoform- and concentration-dependent changes in the activity of ADAM10 were detected with a rank order of apoE2>apoE3>apoE4. At 25ng/ml apoE, ADAM10 activity was significantly lower in the presence of apoE4 compared to apoE2 (40% reduction). Although it did not reach statistical significance, apoE3 showed consistently higher activity than apoE4 at all concentrations. In addition, we also investigated the influence of apoE isoforms on ADAM10 activity via Michaelis Menten kinetics (Figure 4.6B). This revealed that apoE2 demonstrated the greatest impact on ADAM10 activity (EC50 = 0.69ng/ml) followed by apoE3 and apoE4 (EC50 = 16.81ng/ml and 27.17ng/ml, respectively)

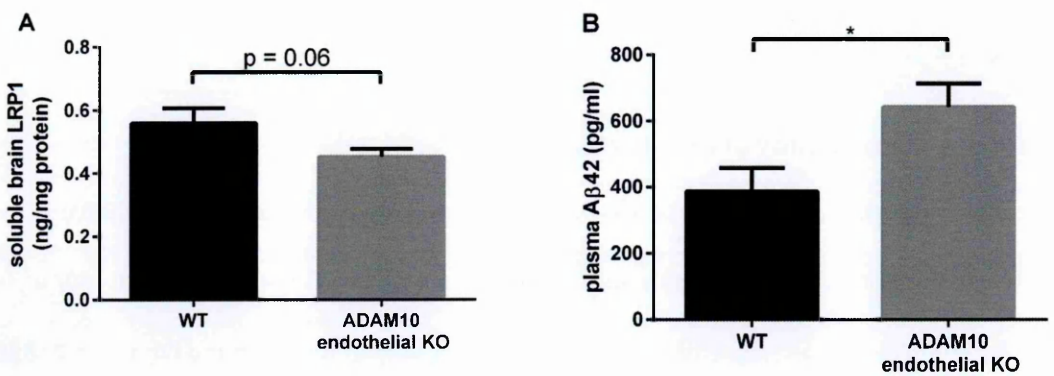

Figure 4.3 : LRP1 shedding in the brain and Aβ clearance across the BBB in ADAM10 endothelial KO and wild-type mice. Human Aβ42 (1mM) was intracranially injected into ADAM10 endothelial KO and wild-type mice 10 minutes before collection of the brain and plasma. The soluble fraction of the brain was probed for (A) LRP1, while the plasma was probed for (B) Aβ42 using ELISAs. Values represent mean ± SEM (n = 9 biological replicates) and are expressed as amount of LRP1 per mg total protein or

amount of Aβ42 per ml. Statistical analyses were performed using an unpaired t-test compared to the wild-type animals.

4.4 Discussion

Dysfunction within the cerebrovascular system is now recognized as a major contributory factor in the development of AD [281–283]. Prior studies have shown that reduced levels of the receptors that transport Aβ in brain endothelia, such as LRP1, results in decreased Aβ clearance across the BBB, elevated Aβ burden in the brain, and aggravated memory and learning deficits [123,282]. Our previous works, and the work of others, shows LRP1 is susceptible to ectodomain shedding by ADAM10 (Chapter 2.3.7) [146]. Therefore, in this chapter, we investigate whether inhibition of ADAM10 was a viable therapeutic strategy to facilitate the clearance of Aβ by blocking the shedding of LRP1.

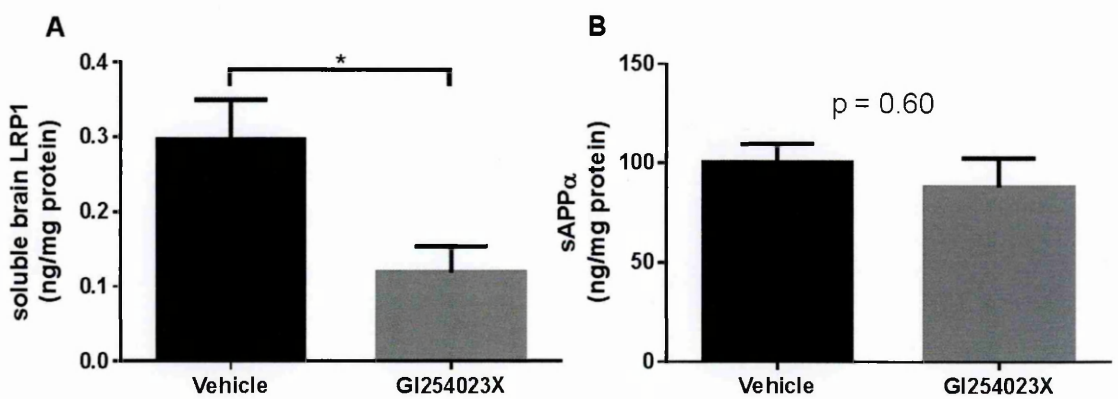

Figure 4.4 : LRP1 shedding and sAPPα levels in the brain following ADAM10 modulation in a mouse model of AD. The ADAM10 inhibitor GI254023X (200mg/kg) or vehicle was administered via intraperitoneal injection once per day for five consecutive days to PSAPP mice (35 weeks of age). One hour after the final injection, the brains were collected and the soluble brain fraction was probed for

(A) LRP1, while the whole brain homogenate was probed for (B) sAPPα using ELISAs. Values represent mean ± SEM (n = 9 biological replicates) and are expressed as the amount of LRP1 or sAPPα per mg of total protein. *p<0.05 as determined by an unpaired t-test compared to vehicle control.

Our initial studies found that inhibition of ADAM10 effectively reduced LRP1 shedding and increased Aβ transit across an *in vitro* model of the BBB. This was supported by our *in vivo* studies in which ADAM10 endothelial KO mice displayed less LRP1 shedding in the brain in conjunction with increased Aβ clearance across the BBB compared to wild-type animals. However, the reductions in the levels of soluble LRP1 in the ADAM10 endothelial KO group did not reach statistical significance, although a strong trend was observed. This may not be all that unexpected as there are number of cell types in the brain that express LRP1 in addition to brain endothelia. As our analytical approach assessed LRP1 levels in the entire soluble fraction of the brain, endothelial-specific changes in LRP1 shedding may be difficult to capture with so many other cells types contributing to the pool of soluble LRP1. Nevertheless, our findings *in vitro* and *in vivo* demonstrate that modulation of the ADAM10 enzyme minimizes LRP1 shedding and facilitates Aβ clearance across the BBB.

As we identified a role for ADAM10 in mediating Aβ clearance across the BBB, we next examined the impact of ADAM10 inhibition on Aβ tissue levels in an animal model of AD. Using an acute 5-day treatment paradigm with the ADAM10-selective inhibitor GI254023X in PSAPP mice, we observed a substantial decrease in LRP1 shedding in the brain compared to vehicle-treated animals, which coincided with a significant increase in plasma Aβ40 levels. We propose that inhibition of ADAM10 facilitated Aβ40 transit from the brain to the periphery (via reduced LRP1 shedding), resulting in the elevated Aβ40 plasma levels we observed. However, no significant changes in plasma Aβ42 levels were detected following GI254023X treatment. A potential explanation for the disparity we observed in the BBB clearance between Aβ40 and Aβ42 may be due to differences in the transport rates for these

species. A recent report indicated LRP1 preferentially clears Aβ40 over Aβ42 [123] and prior studies have shown that the rate of Aβ40 transport across the BBB is more than twice that observed for Aβ42 [96,284]. Nevertheless, the increased levels of Aβ40 in the plasma following ADAM10 inhibition suggest this treatment strategy may be used to facilitate the transit of Aβ from the brain to the periphery.

To examine the impact of ADAM10 inhibition on Aβ levels in the brain, we examined both guanidine soluble and insoluble Aβ following GI254023X treatment in PSAPP animals. While we observed reductions in both soluble and insoluble Aβ40 and Aβ42 in the brain following treatment, none of these effects were statistically significant. It is unclear why this treatment paradigm did not modulate Aβ levels in the brain more effectively. Both our prior work [202] and our current findings demonstrate a strong relationship between brain LRP1 shedding and Aβ clearance across the BBB. In addition, although GI254023X treatment in the PSAPP mice reduced LRP1 shedding in the brain by 60%, this effect did not translate to significant changes in Aβ levels in the brain. It may be that such an acute treatment

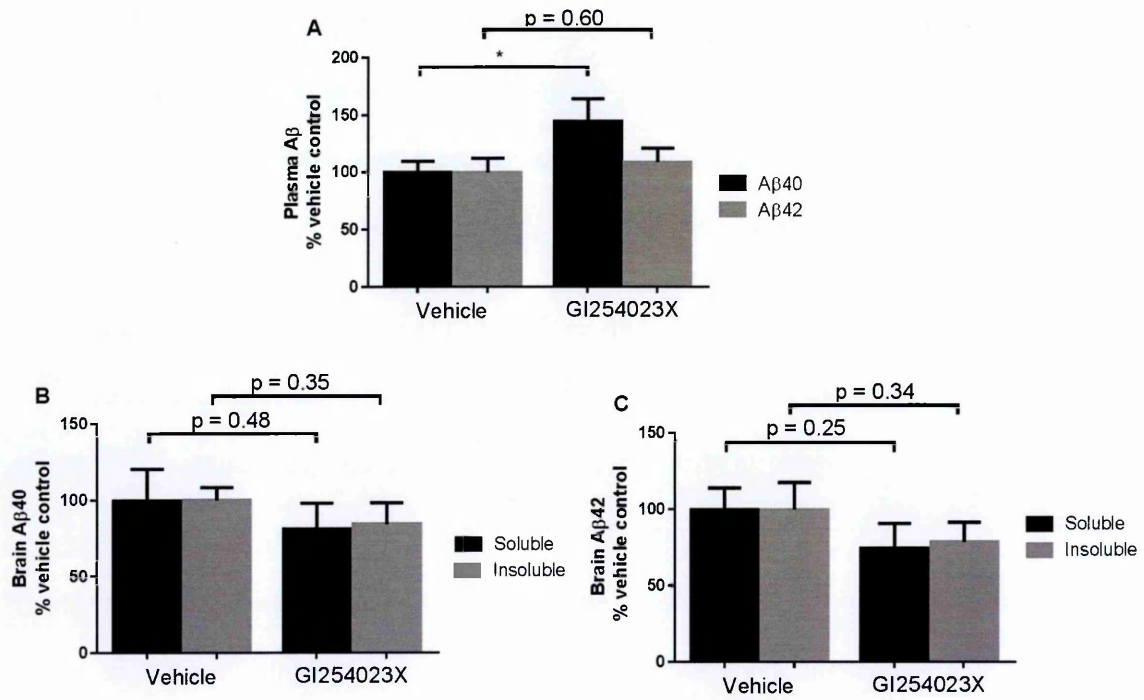

Figure 4.5 : Aβ tissue levels following ADAM10 modulation in a mouse model of AD. The ADAM10 inhibitor GI254023X (200mg/kg) or vehicle (DMSO) was administered via intraperitoneal injection once per day for five consecutive days to PSAPP mice (35 weeks of age). (A)One hour after the final injection, the plasma was collected and probed for Aβ40 and Aβ42 using an ELISA, while the brain was probed for guanidine soluble and insoluble (B) Aβ40 and (C) Aβ42, also using an ELISA. Values represent mean ± SEM (n = 9 biological replicates) and are expressed as the percentage change from vehicle control. Statistical analyses were performed using an unpaired t-test compared to vehicle control.

paradigm (5 days) is not sufficient to demonstrably lower Aβ levels in the brain, and that a more chronic treatment paradigm is necessary. Another possible explanation for our observations is that LRP1 expression is known to be lower in AD patients [100,129] and AD animals, including the PSAPP mice used in the current studies [285]. Exposure of brain vascular cells to high levels of Aβ for a prolonged period has also previously been shown to reduce the expression of LRP1 [129]. In our study, the PSAPP mice were tested at an age when extensive Aβ pathology is present [274,286]. As such, the total LRP1

population may be depleted to such an extent that any improvements in LRP1 shedding to promote Aβ elimination would still prove insufficient. Therefore, this therapeutic approach may be more impactful if used earlier in the disease process when a greater density of LRP1 receptors is available for therapeutic targeting. Therefore, further evaluation of this treatment protocol and the feasibility of targeting LRP1 sheddase enzymes in AD are certainly warranted.

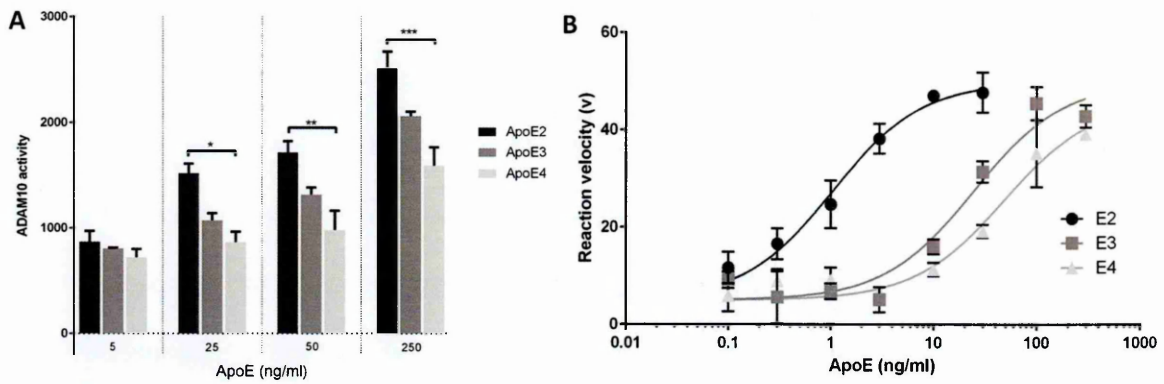

Figure 4.6 : Differential modulation of ADAM10 activity by the apoE isoforms. (A) ADAM10 activity in a cell-free paradigm was significantly modulated by apoE in an isoform and dose dependent manner (apoE2>apoE3>apoE4). Differences between apoE2 and apoE4 were statistically significant at concentrations ≥25ng/ml. For all apoE concentrations, ADAM10 activity was lower in the presence of apoE4 compared to the other isoforms. (B) ApoE isoform-specific influence on ADAM10 EC50 values. ApoE2 demonstrated the greatest influence on ADAM10 activity (EC50 = 0.69ng/ml ± 0.18) in comparison to apoE3 (EC50 = 24.27ng/ml ± 8.04) or apoE4 (52.24ng/ml ± 14.01). Activity data are presented as ADAM10 activity (n=3) ± SEM. Statistical significance was determined by two-way ANOVA followed by Tukey's *post-hoc* analysis. EC50 values were generated using a nonlinear regression curve with reaction velocity and concentration of apoE (EC50 ± SEM n=3 biological replicate). *p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

A primary concern in targeting an enzyme like ADAM10, especially in AD, is the potential impact on other substrates that are metabolised by ADAM10. ADAM10 is one of the α-secretases which processes APP through the non-amyloidogenic pathway, resulting in the formation of sAPPα while at the same time, precluding the production of Aβ peptides. As such, inhibition of this pathway could facilitate Aβ synthesis and potentially exacerbate Aβ burden in the AD brain. Our data suggests this is not the case, as Aβ levels in the brain did not increase upon ADAM10 inhibition, but in fact decreased, albeit modestly. To ascertain whether GI254023X treatment influenced the α-secretase pathway specifically, we measured sAPPα levels in the brain and found no difference between GI254023X-treated animals and the vehicle control group. These data indicate ADAM10 can be modulated to reduce LRP1 shedding in the brain without affecting the α-secretase cleavage of APP. One explanation for this may be the presence of other α-secretase enzymes, which are able process APP when ADAM10 is diminished or absent. To this point, it was previously found that sAPPα formation was preserved in fibroblast cells derived from ADAM10-deficient animals [287]. It has also been reported that other members of the α-secretase family such as ADAM9 and ADAM17 are able to compensate for reductions in ADAM10 activity [287,288]. Alternatively, another report did observe a significant change in sAPPα production in primary neurons when ADAM10 was absent, suggesting a lack of compensation by other α-secretases [64]. Data from the *in silico* BBB permeability models suggests that the permeability of GI254023X is marginal, with 50% of the tests returning a non-BBB permeable prediction. Therefore, it is possible that GI254023X is not able to reach neurons, where the majority of APP processing is located, at a high enough concentration to influence the non-amyloidogenic processing of APP[289]. Nevertheless, our studies demonstrate that targeting the ADAM10 enzyme can effectively reduce LRP1 shedding in the brain without impacting APP proteolysis.

In addition to reducing shedding of LRP1 and improving clearance of brain Aβ, inhibition of ADAM10 at the BBB may have additional benefits due to its association with other cerebrovascular related pathologies. In particular, increases in ADAM10 expression due to ADAM10 polymorphisms have been

linked with risk factors for AD such as atherosclerotic cerebral infarctions [264]. In addition, ADAM10 is also capable of cleaving collagen IV which is a major component of the basement membrane of the vasculature [290,291]. A loss of normal collagen function as a result of increased ADAM10 has been demonstrated to lead to increased vascular permeability while also reducing transmigration of cells due to disruption of E-cadherin function [292,293]. Finally, on a cellular level, cell associated LRP1 has been shown to reduce the delivery of apoE to lysosomes, thus preventing apoE degradation [176]. As apoE expression inversely correlates with Aβ load and therapies directed at increasing its expression have shown some success in animal models[180,182], protecting LRP1 at the cell surface may also facilitate increased apoE recycling and therefore reduce amyloid levels. In addition, it is also possible that inhibition of ADAM10 protects LRP1 from shedding in the periphery and therefore may also facilitate increased hepatic clearance of Aβ [196].

As APOE genotype is an important factor to consider when investigating the mechanistic pathways that may be involved in AD as a result of its strong association with the risk of developing AD, especially with the APOE4 genotype. In our study, ADAM10 activity was found to be reduced in the presence of apoE4 compared to the other apoE isoforms. In addition, we also identified differences in the EC50 of ADAM10 by each apoE isoform (apoE2>apoE3>apoE4). Prior work investigating this apoE-ADAM10 interaction have primarily focused on the processing of APP through the non-amyloidogenic pathway and, in doing so, failed to detect apoE-dependent differences in sAPPα levels *in vitro* [270]. However, our study utilised lipidated apoE, which is likely a better representation of apoE under normal physiological conditions than de-lipidated recombinant apoE. Recent studies have indicated that apoE function is dependent on the lipidation status of apoE; with poorly lipidated apoE demonstrating different binding affinities to Aβ while also promoting increased amyloidogenesis in mouse models of AD [168–170]. However, the impact of apoE on the activity of ADAM10 (apoE2>apoE3>apoE4) is not consistent with the findings in Chapter 2 with regards to the shedding of LRP1 (apoE2≤apoE3<apoE4). This suggests that the proteolytic shedding of LRP1 by ADAM10 is not influenced by the apoE isoforms. Therefore, the

apoE dependent shedding of LRP1 outlined as in Chapter 2 appears to be driven by another of the sheddases. However, while the apoE dependent activity profile of ADAM10 suggests it is not the mechanism responsible for the apoE-mediated shedding of LRP1, it may prove to be of particular interest with regard to the processing of APP by α-secretases down the non-amyloidogenic pathway. For instance, this data suggests that the apoE2 isoform increases the activity of ADAM10 which may result in the diversion of more of APP down the non-amyloidogenic pathway and away from Aβ production when compared to the apoE4 isoform.

In summary, in this chapter we show that modulation of the ADAM10 enzyme can effectively reduce LRP1 shedding and promote Aβ transport out of the brain. After treatment of AD mice with an ADAM10 inhibitor, we observed increased levels of Aβ40 in the plasma, suggesting that BBB clearance of Aβ40 was elevated. However, the reductions witnessed in the levels of brain Aβ40 and Aβ42 failed to reach statistical significance. This suggests that a more chronic treatment paradigm may be necessary to observe demonstrable changes in Aβ brain burden. Nevertheless, while further interrogation of this therapeutic approach is necessary, our findings indicate LRP1 sheddases can be targeted to facilitate Aβ elimination through the BBB, providing a novel therapeutic strategy to mitigate Aβ accumulation in the AD brain. While our findings in the previous chapters indicated an isoform-dependent role for apoE in LRP1 shedding and Aβ-BBB clearance, our observed effects of apoE on the activity of ADAM10 do not explain the apoE dependent shedding of LRP1 described in Chapter 2. This suggests that while ADAM10 is capable of shedding LRP1 and has an impact on the clearance of Aβ through the BBB, it is not responsible for the apoE-mediated shedding of LRP1 outlined in Chapter 2. Therefore, Chapter 5 will assess the impact of another sheddase, MMP9, on LRP1 shedding and the clearance of Aβ through the BBB to further elucidate the mechanisms behind the isoform-specific effects of apoE we observed prior Chapters.

Chapter 5 : The influence of MMP9 on LRP1 shedding and clearance of Aβ across the BBB

5.1 Introduction

We previously demonstrated that inhibition of the LRP1 sheddase ADAM10, with the aim of increasing the population of cell associated LRP1, is a viable method for increasing the clearance of Aβ through the BBB (Chapter 4). However, a comparison of the apoE isoform dependent activity profile of ADAM10 (Figure 4.6) and the apoE isoform dependent shedding of LRP1 (Figure 2.4) revealed that it may not be the main driving factor behind this mechanism. In light of this, we moved our focused to another of the LRP1 sheddases, the matrix metalloproteinase 9 (MMP9), in order to assess its impact on the BBB clearance of Aβ and any interactions it may have with apoE. [147]. The matrix metalloproteinases (MMPs) are a family of 28 membrane-bound and secreted calcium-dependent zinc-containing endopeptidases. They are involved in a diverse array of processes including the degeneration of the extracellular matrix components (ECM), remodelling of tissues, shedding of cell surface markers and processing of several signalling molecules in addition to their critical functions in the inflammatory response [294]. Due to the multitude of pathways in which they are involved, MMP activity is highly regulated at both the transcriptional level and by inhibition by tissue inhibitors of metalloproteinases (TIMPs), which bind competitively to their active site [295,296]. However, despite the multiple regulatory mechanisms that exist to keep the activity and expression of the MMPs in check [296,297], dysfunctional regulation or altered expression of MMPs is implicated in the pathogenesis of several neurodegenerative disorders such as AD, Parkinson's Disease and Multiple Sclerosis [298–300].

Of the members of the MMPs family, MMP9 is particularly associated with vascular damage, dysfunction of the blood-brain barrier (BBB) and AD. Originally termed type IV collagenase or gelatinase B, MMP9 is synthesised by a diverse array of cells in the central nervous system, such as astroglia, microglia and neurons [301]. Activation of MMP9 is achieved through a protease cascade initiated by plasmin which

results in the cleavage of pro-MMP9 to a 82kDa enzymatically active enzyme [302]. MMP9 contains a domain unique to this species consisting of three repeats of fibronectin type II which is essential for binding to denatured collagen or gelatine [303]. As such, increased MMP9 expression results in tissue remodelling and the breakdown of the basement membrane [304,305]. Activation of MMP9 in the vasculature is closely linked to the activity of tissue plasminogen activator (tPA), which increases MMP9 production under hypoxic or ischemic conditions through signalling via the light chain domain of LRP1 [306,307]. In addition to the studies outlined in Chapter 2, there are also several studies that have demonstrated a role of MMP9 in the regulation of LRP1 expression and function [147,229]. These suggest that MMP9 has a prominent role in the ectodomain shedding of LRP1 which results in the formation of soluble LRP1. Therefore, it is possible that the AD associated cerebrovascular dysfunction and defective BBB clearance mechanisms may have MMP9 activity as a common factor. In support of this, AD patients were found to have significantly higher MMP9 levels in the plasma when compared to control individuals [298]. In addition, exposure of mouse cerebral endothelial cells and rat microvessels to Aβ induced expression of MMP9 and elevated activity [308,309]. Interestingly, there is also some evidence to suggest that elevated MMP9 levels in the plasma may be restricted to types of AD that have a vascular component, with MMP9 expression significantly higher in the plasma of patients with vascular AD when compared to non-vascular AD [310]. In addition to changes in activity and expression of MMP9, reduced expression of the endogenous inhibitors TIMP1 and TIMP2 have also been identified [311]. This suggest that the endogenous inhibitory mechanisms that help regulated MMP9 activity may also be dysfunctional. In support of this, the ratio of MMP9 and TIMP1 in the plasma was found to be a strong predictor for differentiating vascular AD from non-vascular AD [312]. Vascular components in Alzheimer's disease are common, with approximately 80% of autopsied cases showing deposition of Aβ in the cerebral vessels, termed cerebral amyloid angiopathy (CAA) [308,313]. The presence of CAA is associated with both higher expression and activity of MMP9 [308].

Aside from the vascular associated pathologies linked with MMP9, increased MMP9 activity has also been detected in the frontal cortex of AD patients, where it was found to have a strong inverse correlation with the Mini Mental State Exam (MMSE) score [314]. This suggests that increased MMP9 expression or activity may even have a direct deleterious effect on the function of neurons and cognition. Similarly, MMP9 null mice demonstrate lower levels of neuronal pathology compared to wild type mice after intracerebral haemorrhage as well as reduced microglia and macrophage activation [315]. In addition, MMP9 inhibition has been shown to reduce Aβ induced cognitive deficits and neurotoxicity in a mouse model of AD [316].

As previously discussed, APOE4 constitutes the strongest genetic risk factor for AD, with possession of one or two APOE4 alleles increasing the chance of developing AD by 4- and 15- fold, respectively when compared to APOE3 homozygous individuals [160,162]. The ectodomain shedding of LRP1 outlined in Chapter 2, was also found to be apoE isoform dependent, suggesting that the proteases involved in this process must be sensitive to the presence of the different apoE isoforms. In Chapter 4, we demonstrated that while ADAM10 is capable of causing the shedding of LRP1, it may not be the primary driver as its apoE activity profile does not match the apoE mediated shedding demonstrated in Chapter 2. Therefore, this chapter will investigate if MMP9 is the driver of the apoE isoform-mediated shedding of LRP1 and whether blocking MMP9 is a viable therapeutic strategy in AD.

5.2 Methods

5.2.1 Animals

APOE-targeted replacement (APOE-TR) mice (4-6 months of age) were used in this study. These mice are from a C57BL/6 background and retain the endogenous regulatory sequences for the physiological expression of apoE while expressing the human apoE variants in the absence of the mouse apoE. Mice

were group housed in a temperature and humidity controlled environment on a 12 hour light/dark cycle with free access to food and water. All the experiments involving mice were approved by the Institutional Animal Care and Use Committee of the Roskamp Institute.

5.2.2 Preparation of Aβ42 peptides

Aβ42 peptides were prepared in an identical method as previously described in Chapter 2 (section 2.2.3).

5.2.3 *In vitro* LRP1 shedding and MMP9 production

Human Brain Microvessel endothelial cells (HBMECs) (ScienCell, USA) were seeded at approximately 50,000 cells per cm^2 into fibronectin-coated 6-well plates [216]. When 90% confluent, cells were treated with recombinant MMP9 at 0-250ng/ml (EMD Millipore, USA) to observe shedding of LRP1, or with Aβ42 (0-20μM) to assess any change in MMP9 levels in the media. DMSO concentration was <0.1% in all treatment wells. Levels of soluble LRP1 and MMP9 were assessed in the media via LRP1 ELISA (Cedar Lane Labs, USA) or MMP9 ELISA (Invitrogen, USA) after the 48 hour incubation period.

5.2.4 Influence of MMP9 on transit of Aβ across an *in vitro* model of the BBB

The impact of MMP9 on Aβ42 transcytosis was assessed using an *in vitro* BBB model previously described by our group [216]. HBMECs were seeded onto fibronectin-coated 24-well membrane inserts forming a polarised monolayer representing the BBB. Aβ transcytosis across the BBB was assessed by exposing the basolateral side ("brain") to 2μM fluorescein-Aβ42 and treating the apical side ("blood") with the MMP9-selective inhibitor SB-3CT (0.1-10μM). The levels of DMSO were kept under concentrations previously shown to have no effect on the integrity of the membrane [216]. After 60 minutes of incubation at 37°C, samples were collected from the apical compartment to assess the basolateral-to-apical transcytosis of fluorescein-Aβ across the BBB model. To ensure the integrity of the barrier was intact, the basolateral-to-apical permeability of a paracellular marker, 10kDa lucifer yellow dextran, across the BBB model was examined for each study. The levels of fluorescein-Aβ in the apical

compartment were assessed via fluorescence at 485/515 Ex/Em using BioTek Synergy HT microplate reader.

5.2.5 Collection and enrichment of human lipidated apoE

Lipidated apoE was collected as previously described in Chapter 2 (section 2.2.4).

5.2.6 Impact of apoE isoforms on MMP9 activity

The effect of apoE isoforms on MMP9 activity was assessed in a cell-free paradigm utilising a fluorescent substrate as per the manufacturers' instructions (Anaspec, USA). Briefly, recombinant MMP9 (5nM) was incubated in the presence of lipidated apoE2, 3 and 4 (0-250ng/ml) and the fluorescent substrate for 1 hour at 37°C before detection of fluorescence. To determine whether the substrate was metabolised by apoE itself, fluorescent substrate was incubated with various concentrations of apoE alone in the absence of MMP9. These values were used as background controls for each apoE isoform-MMP9 treatment combination. Fluorescence was measured at 340/490 Ex/Em using BioTek Synergy HT microplate reader.

5.2.7 ApoE isoform dependent shedding of LRP1 by MMP9 in isolated APOE-TR mouse cerebrovasculature

The cerebrovascular fractions were isolated from APOE-TR mouse cortices using a technique previously described by our group [202]. Briefly, using a Dounce homogenizer, the tissue was homogenized in HBSS on ice. The homogenized tissue was diluted 1:1 with 40% dextran followed by centrifugation at 6000g for 15 minutes at 4°C. The soluble fraction and parenchymal pellets were discarded leaving the remaining cerebrovasculature. These were treated with MMP9 (0-250ng/ml) for 3 hours at 37°C and the extracellular solution probed for soluble LRP1 using an LRP1 ELISA (Cedar Lane Labs, USA). Unfortunately, due to limited numbers and difficulties in mouse breeding (personal communications LaDu), only APOE2 and APOE4 mice were available for use in this study.

5.2.8 Inhibition of MMP9 and LRP1 shedding *in vivo*

APOE-TR mice were administered vehicle (25% DMSO, 65% PEG400, 10% water) or the MMP9 selective inhibitor, SB-3CT (25mg/kg) via intraperitoneal injection. This dose has previously been demonstrated to effectively attenuate MMP9 function [305,309,317]. 45 minutes after the i.p. injection, the mice were intracranially injected with human Aβ42. Ten minutes after the Aβ42 injection, the mice were euthanatized and the plasma and brain were collected. Soluble brain fractions were isolated from mouse whole cortex samples using a technique as previously described [202]. The parenchymal cerebrovascular pellets were discarded and the levels of soluble LRP1 were measured in the soluble fraction by LRP1 ELISA (Cedar Lane Labs, USA). Additionally, the levels of the intracranially injected human Aβ42 was measured in the plasma by ELISA (Invitrogen, USA). Unfortunately, due to difficulties in mouse breeding (personal communications LaDu), only APOE3 and APOE4 mice were available for use in this study.

5.2.9 Statistical Analysis

Statistical analyses were performed using a one- or two-way ANOVA followed by Tukey's *post-hoc* analysis (GraphPad Prism 5, GraphPad Software Inc., USA). Unless otherwise indicated, all n values represent technical replicates.

5.3 Results

5.3.1 MMP9 induces shedding of LRP1 *in vitro*

To assess whether MMP9 is capable of inducing shedding of LRP1 *in vitro*, we exposed HBMECs to increasing concentrations of MMP9 for 48 hours (Figure 5.1A). Both 100ng/ml and 250ng/ml MMP9 significantly increased the shedding of LRP1 as detected in the cell culture media. The highest of these concentrations increased the levels of soluble LRP1 by 2-fold.

5.3.2 MMP9 release is induced by Aβ in HBMECs

Exposure of HBMECs to Aβ42 for 48 hours demonstrated a significant increase in the expression of MMP9 in a dose dependent manner (Figure 5.1B). Treatment of HBMECs with concentrations higher than 10μM Aβ42 increased the expression of extracellular MMP9 in the media by at least 6-fold when compared to the control.

5.3.3 Influence of MMP9 on transit of Aβ across an *in vitro* model of the BBB

The transit of fluorescein-Aβ42 across an *in vitro* model of the BBB was increased by inhibition of MMP9 (Figure 5.2). A dose dependent increase in the basolateral to apical transport of fluorescein-Aβ was observed with the MMP9 inhibitor SB3-CT with greatest increase in BBB transit at the highest concentration (2-fold increase at 10μM). In addition, treatment did not significantly increase the amount of 10kDa lucifer yellow dextran in the apical compartment, suggesting the integrity of the confluent cell monolayer was not impacted by treatment.

5.3.4 ApoE dependent MMP9 activity

The activity of MMP9, as measured by cleavage of fluorescent substrate, was inhibited in a dose and apoE isoform manner (apoE2>apoE3>apoE4) (Figure 5.3). At 25ng/ml apoE, both apoE2 and apoE3 significantly reduced the cleavage of the substrate to approximately 70 and 80% of the control respectively. Inhibition was greatest that the highest dose of apoE (250ng/ml) where apoE2, apoE3 and apoE4 significantly reduced cleavage of the MMP9 substrate to 20, 30 and 50% of the control.

5.3.5 ApoE dependent shedding of LRP1 in an ex vivo cerebrovascular preparation

In order to assess the influence of MMP9 on the shedding of LRP1 in an apoE environment, MMP9-mediated shedding of LRP1 was assessed in an *ex vivo* preparation of the cerebrovasculature from APOE-TR mice (Figure 5.4). Levels of soluble LRP1 were highest in the vasculature from APOE4 after MMP9 treatment, with the vasculature from APOE2 mice showing the lowest levels. Both genotypes demonstrated higher levels of soluble LRP1 with MMP9 treatment compared to untreated controls.

5.3.5 *In vivo* shedding of LRP1 and BBB clearance of Aβ in APOE-TR mice

To assess the impact of MMP9 on the shedding of LRP1 *in vivo*, MMP9 activity was inhibited using an acute SB-3CT treatment paradigm (Figure 5.5A). This resulted in a significant reduction in the levels of soluble LRP1 in the APOE4 mice (>50% reduction). In addition, clearance of Aβ through the BBB in the SB-3CT treated APOE4 mice was significantly higher than in the APOE4 naive mice (Figure 5.5B). The BBB clearance of Aβ in the treated APOE4 mice reached a level comparable to that seen in the untreated APOE3 mice.

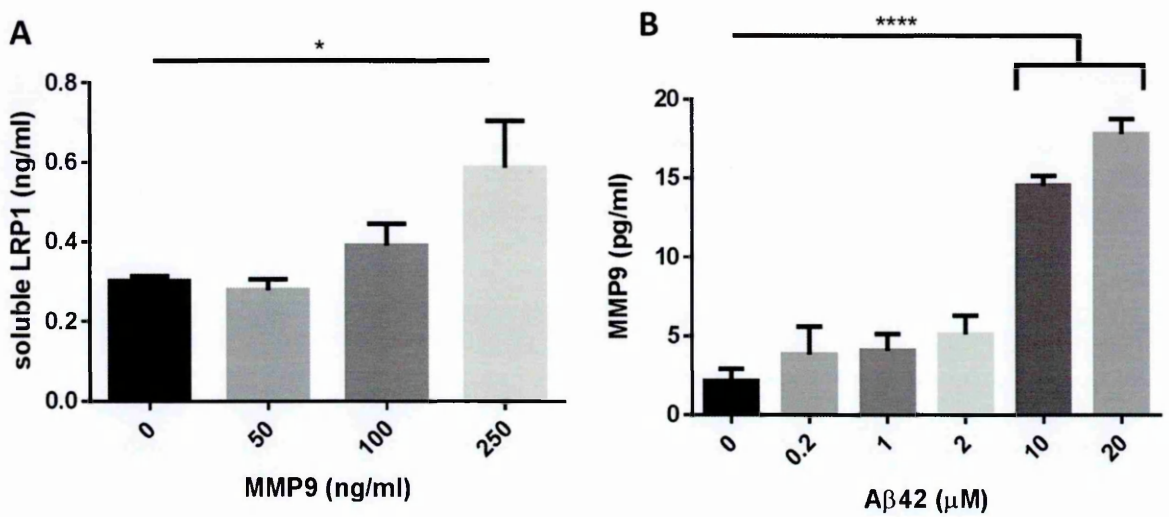

Figure 5.1 : The influence of MMP9 on the shedding of LRP1 in HBMECs. (A) HBMEC monolayers were exposed to active MMP9 (0-250ng/ml) for 48 hours at 37°C. LRP1 shedding was assessed by examining LRP1 content in the extracellular media using an LRP1 ELISA. (B) MMP9 levels were increased in the media of HBMECs after treatment with Aβ42 (0-20μM) in a dose dependent manner. MMP9 levels were assessed by examining MMP9 content in the extracellular media using an MMP9 ELISA. Values represent mean ± SEM (n = 3) and are expressed as ng of extracellular protein per ml of media. *p < 0.05; ****p<0.0001 as determined by one-way ANOVA followed by Tukey's post-hoc analysis.

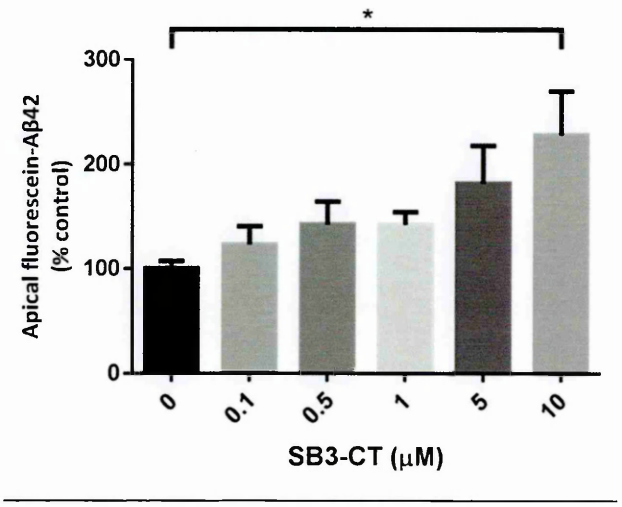

Figure 5.2 : Fluorescein-Aβ42 transit across an *in vitro* BBB model following MMP9 inhibition. Fluorescein-Aβ42 (2μM) was exposed to the basolateral ("brain") side of the *in vitro* BBB model, while various concentrations of the MMP9 inhibitor SB3-CT (0-10μM) were exposed to the apical ("blood") compartment. Following incubation at 37°C, samples were collected from the apical compartment at 60 minutes to assess the basolateral-to-apical transcytosis of fluorescein-Aβ42 across the BBB model. Values represent mean ± SEM (n = 4) and are expressed as the percentage change from control conditions. *p<0.05 as determined by one-way ANOVA followed by Tukey's *post-hoc* analysis.

5.4 Discussion

Dysfunctional regulation of MMP9 in AD has been shown to have multiple implications in both vascular and neurological health as a result of the diverse array of processes in which it is involved. For example, high levels of MMP9 is associated with hypoxic or ischemic conditions, reduced performance in cognitive tests and breakdown of basement membranes [298,304–307,314]. There are also indications that MMP9 may be involved in pre-clinical AD, with increased expression of MMP9 detected in the CSF of healthy individuals who have an AD-supportive CSF biomarker pattern [318,319].

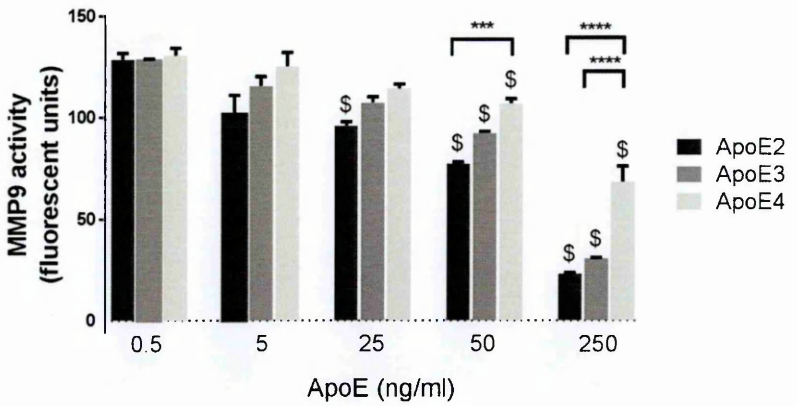

Figure 5.3 Differential modulation of MMP9 activity by the apoE isoforms. MMP9 activity was significantly modulated by apoE in an isoform and dose dependent manner (apoE2>apoE3>apoE4). Differences between apoE2 and apoE4 were statistically significant at concentrations ≥25ng/ml. Values represent mean ±SEM (n = 3) and are expressed as fluorescent units. Statistical significance was determined by two-way ANOVA followed by Tukey's *post-hoc* analysis. **** $p<0.0001$; $^{\$}p<0.05$ compared to the lowest dose of apoE.

Although the precise cause and trigger of this altered MMP9 activity is unknown, it is clear that strategies targeting MMP9 may be beneficial on multiple fronts with regards to AD pathology. The current study sought to elucidate the MMP9 driven mechanisms that may contribute to the effect of apoE on the clearance of Aβ through the BBB as described in Chapter 2. Initially, we confirmed the ability of MMP9 to cleave the full length LRP1 in HBMECs. Interestingly, the levels of MMP9 that induce shedding of LRP1 in both our *in vitro* and *ex vivo* studies are similar to the levels witnessed in the plasma of AD patients [298]. The direct involvement of MMP9 in the clearance of Aβ was demonstrated in an *in vitro* BBB model with increasing concentrations of MMP9-selective inhibitor improving the transcytosis of Aβ across a polarised membrane. We also demonstrated that the expression of MMP9 itself can be

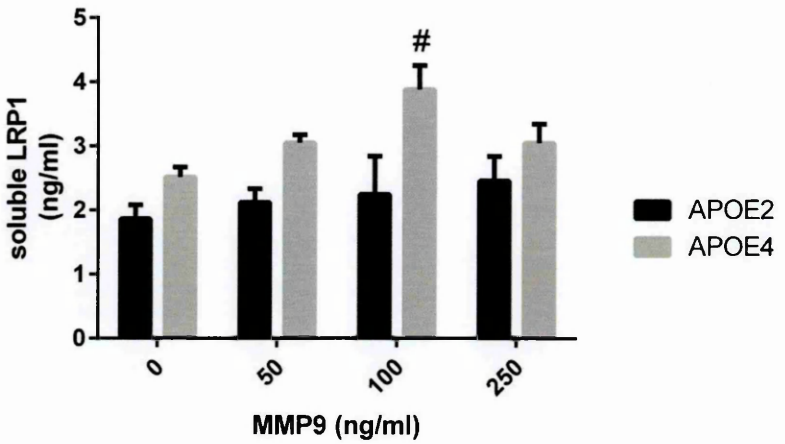

Figure 5.4 Shedding of LRP1 by MMP9 in an *ex vivo* preparation of APOE-TR mouse cerebrovasculature. Induction of LRP1 shedding by MMP9 (0-250ng/ml) in the APOE-TR mouse cerebrovasculature increased soluble LRP1 in an APOE dependent manner (APOE2<APOE4). MMP9 increased the levels of soluble LRP1 in all genotypes with APOE4 mice demonstrating the largest increase between control and MMP9 treated at 100ng/ml MMP9. Values represent mean ±SEM (n = 4 biological replicates) and are expressed as ng of LRP1 protein per ml. ****p<0.0001 as determined by two-way ANOVA followed by Tukey's *post hoc* analysis.

induced in endothelial cells by exposure to Aβ, an important factor to consider with regards to AD and the amyloid cascade. We hypothesise that there be a may feed-forward mechanism, whereby expression of MMP9 is increased due to the high levels of Aβ, resulting in reduced clearance of Aβ and therefore, more stimulus for MMP9 release. The activity of MMP9 is also dependent on the apoE isoform present. While all apoE isoforms demonstrated inhibitory-like behaviour towards MMP9, apoE4 demonstrated significantly lower ability to inhibit activity compared to the other apoE isoforms. The lack of inhibition of MMP9 in the presence of apoE4 may be the mechanism responsible for the increased shedding of LRP1 and the lowered BBB clearance of Aβ as demonstrated in the APOE4-TR mice (Chapter

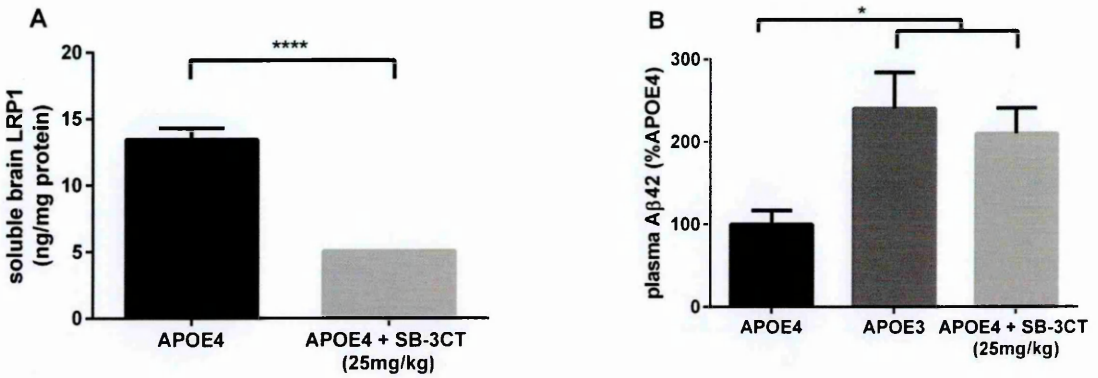

Figure 5.5 The influence of MMP9 on LRP1 shedding and Aβ clearance through the BBB in APOE-TR mice. (A) Treatment of APOE4 mice with the MMP9 inhibitor SB-3CT (25mg/kg) reduced the shedding of LRP1 and (B) increased the clearance of Aβ through the BBB, reaching approximately the same levels as those in naive APOE3 mice. Values represent mean ±SEM (n = 4 biological replicates) and are expressed as ng of LRP1 protein per ml or % control Aβ42 in plasma. *p<0.05, ****p<0.0001 asdetermined by one-way ANOVA followed by Tukey's *post hoc* analysis or Student's t-test.

2). As LRP1 is the most efficient transporter of Aβ when compared to other members of the lipoprotein group, it seems plausible that this reduction in transport occurs as a result of the increased MMP9 mediated shedding of LRP1 in the APOE4 mice and not through changes in other lipoprotein receptor function [131]. The apoE isoform effect was also witnessed *ex vivo*, with the cerebrovasculature from APOE2 mice showing significantly less LRP1 shedding than APOE4 cerebrovasculature after MMP9 challenge. Interestingly, the level of Aβ BBB clearance in the APOE4 treated mice was approximately the same as those observed in naive APOE3 mice. These findings suggest MMP9 inhibition can overcome the inefficiency of the apoE4 isoform and can facilitate Aβ BBB clearance to such a degree that it reaches levels comparable to that observed for the APOE3 genotype.

To date, there has been little investigation into the relationship between the apoE isoforms and MMP9. However, a recent study observed that the apoE2 and apoE3 isoforms reduce the levels of inflammatory molecules which are associated with the activation of MMP9, whereas apoE4 showed little effect. In addition, these effects were found to be mediated through LRP1 signalling [309]. Later studies by the same group identified that reductions of LRP1 in endothelial cells and pericytes was associated with an increased expression of MMP9 in APOE3 and APOE4 individuals with AD when compared to controls [320]. In combination, these findings, and those from this study, strongly implicate MMP9 in the mechanisms that drive the APOE dependent risk factors for AD. In addition, it appears that the impact of MMP9 is multifaceted, seemingly directly altering activity and also altering signalling the cascades through LRP1.

While there have been some reports outlining the beneficial aspects of MMP9 in AD, namely its ability to degrade Aβ plaques as well as Aβ monomers [321], pathological expression of MMP9 results in both structural and functional damage to the cerebrovasculature and BBB clearance mechanisms. It has now been demonstrated that aggregated Aβ plaques do not correlate well with the neuronal apoptosis and synaptic dysfunction seen in AD and that the soluble oligomeric species are the main driver of Aβ toxicity [20,25,26]. Therefore, the ability of MMP9 to degrade plaques, which was previously identified as a potential benefit with regards to AD pathology, may be inconsequential to the symptomatology of AD. In addition, MMP9 mediated degradation of the Aβ40 and Aβ42 monomers is significantly less efficient than other MMP species, and is therefore probably not the primary proteolytic enzyme involved in this process [322]. This suggests that increasing the expression of MMP9 with the aim of degrading Aβ is ineffectual, and therefore probably has little positive effect. Furthermore, the inhibition of MMP9 activity in AD patients may have several beneficial outcomes in addition to improving the LRP1-mediated transport of Aβ through the BBB as outlined in this study. For example, it has been demonstrated that inhibiting MMP9 reduces Aβ-induced cognitive deficits and neurotoxicity in mouse models [316,323], lowers microglial activation [315], and can protect the vasculature and BBB tight

junctions from damage and degradation [309,317,324]. In addition, proteolysis of tau by MMP9 facilitates the formation of tau oligomers and increases aggregation whereas cleavage by other members of the MMP family appears to occur in sequences that are required for fibril formation [325]. Therefore, MMP9 inhibition may also reduce AD-related tau pathology.

Previously, the inhibition of MMPs as a therapeutic target has been hampered by dose-limiting toxicity and insufficient clinical benefit, probably as a result of the diverse roles of in which MMPs play in cellular homeostasis [326–328]. However, selective targeting of MMP9 in rats using antibody based approaches has been shown to inhibit its activity and resulted in none of the musculoskeletal pathologies that are associated with broad spectrum inhibition of MMPs [329,330]. Nonetheless, questions still arise regarding the effect of the inhibition MMP9 on memory function and neurogenesis. It has previously been demonstrated that MMP9 function is required for the completion of LTP, with genetic and pharmacological blockage of MMP9 function in mice causing deficits in long term potentiation and hippocampal-dependent memory tasks which could be completely reversed with administration of active MMP9 [331,332]. In contrast, blockade of MMP9 has also been demonstrated to improve the memory deficits associated with Aβ [316]. Therefore, inhibition of MMP9 may still prove to be particularly beneficial in an AD environment with high levels of Aβ.

In summary, the data outlined in this chapter suggests that the apoE dependent clearance of Aβ through the BBB may be due to a direct inhibitory interaction of apoE on MMP9 activity. In addition, the increased levels of MMP9 detected in AD may be the result of Aβ driven expression of MMP9 in several cell types, including endothelial cells. This chapter builds on the previously established pathological mechanisms of MMP9 in AD by adding a potential MMP9 dependent and apoE modulated mechanism that increases LRP1 shedding and lowers Aβ clearance through the BBB. It has recently become possible to target MMP9 selectively, thereby opening the door to inhibition of MMP9 as a potential therapeutic target. The ability of this approach to be beneficial in multiple areas of AD pathology, including the

facilitation of BBB clearance of Aβ as outlined in this chapter, makes it an attractive target that certainly warrants further investigation.

Chapter 6 : Discussion and conclusions

6.1 Overview

Alzheimer's disease (AD) is a neurodegenerative disease characterised by a progressive cognitive decline. While deficits in short term memory are often the initial symptoms reported, as the disease progresses, problems with language, loss of motivation, changes in behaviour and disorientation also occur. The clinical diagnosis of AD relies on a battery of tests including the Mini-Mental Status Exam (MMSE), neurological, psychiatric examinations, medical history, and recently developed neuroimaging techniques [7]. However, definitive confirmation of an AD diagnosis still relies on post-mortem pathological analyses based on the localisation of extracellular Aβ plaques and NFTs in the hippocampus, amygdala, cerebral cortex [8,9].

Patients who develop AD have an average life expectancy of 7 to 10 years after diagnosis [333]. The current therapeutic strategies are only palliative and, at best, produce only temporary mild benefits to cognition. As a result prevalence and incidence projections for AD indicate that the number of cases will continue to grow, with 7.7 million new cases every year [334]. The greatest increases are expected to occur in developing countries as a result of their fast growing elderly populations. According to the world health organisation, the total number of people affected by dementias worldwide, of which AD is the most common form, is expected to double every 20 years and reach approximately 115 million individuals by 2050. In addition, it has been estimated that by 2050, 22% of the world population will be over 60 and at high risk of developing AD. Currently, the cost of treating and caring for patients with dementias worldwide is proposed to be at least £450 billion and is expected to rise in line with the increasing prevalence. For example, in Europe, the cost of dementia treatment is expected to rise by as much as 43% between 2008 and 2030 [335]. This increasing economic and social burden as a result of AD is further compounded by the lack of an immediate solution. Therefore, a better understanding of

the processes involved in the aetiology and pathology of AD is essential if the huge burden of this disease is to be addressed through the development of novel therapeutic strategies.

One of the most widely accepted theories put forth to explain the underlying pathology of AD is the amyloid cascade hypothesis. This hypothesis posits that the deposition of Aβ in the brain is a crucial step leading to the development of AD and the associated progressive cognitive decline. While this hypothesis has been altered many times since its inception, the current consensus is that soluble Aβ species are the main pathological driver for AD [336]. The accumulation of soluble Aβ species has been found to strongly correlate with the extent of neurodegeneration as well as the severity of the cognitive symptoms of AD [3,4]. Blocking production of Aβ has historically been a popular strategy for therapeutic intervention in AD. The primary targets for this approach are the β- and γ-secretases which produce Aβ through the processing of APP through the amyloidogenic pathway. However, developing viable therapeutics for AD through the pharmacological modulation these enzymes has proved challenging, with clinical trials either failing to reach their clinical endpoint or being aborted due to adverse reactions [40–42,337]. Regardless, the feasibility of this approach as a therapeutic avenue for AD will be further informed after the completion several BACE inhibitor clinical trials over the next few years [338,339] . Similarly, other approaches, such as the active and passive antibody based approaches which aim to either stimulate production of anti-Aβ antibodies in the periphery or increase the uptake of Aβ into phagocytic cells, have also met with mixed or disappointing results as well as considerable side effects [52,53,55,56,340]. With the high failure rate of AD clinical trials and the enormous projected increase in cases of AD in the future, there is a desperate need for effective disease modifying therapeutics that have the capacity to prevent, or at the very least, slow disease progression [12]. In light of this, the pursuit of new avenues of therapeutic intervention is of the utmost importance if the worldwide burden is to be alleviated.

There is mounting evidence that the accumulation of Aβ in the brain is not due to an increase in Aβ production, but rather a reduced capacity to eliminate Aβ from the brain [75,177]. More specifically, the neurovascular hypothesis of AD proposes that the accumulation of Aβ in the brain and the resulting Aβ pathology occurs as a result of dysfunctional clearance mechanisms for Aβ through the BBB [341]. Interestingly, these Aβ BBB clearance mechanisms appear to be dependent on the apoE isoforms, suggesting that this interaction may be one of the factors that increases the risk of developing AD in individuals with an APOE4 allele [177,179]. In this study, we have proposed several mechanisms to describe our observed effects of apoE on the clearance of Aβ through the BBB. The purpose of this thesis was to interrogate the potential mechanisms involved in this interaction and to identify areas in which this process could be leveraged to restore the clearance of Aβ through the BBB and therefore mitigate the accumulation in of Aβ brain.

6.2 Summary of findings

Clearance of Aβ through the BBB requires the uptake and the subsequent transcytosis of Aβ through the cerebrovasculature. This involves the rapid initial internalisation of Aβ as well as efficient trafficking through the endosomal compartments. Approximately 25% of Aβ clearance from the brain is attributed to clearance through the BBB which, according to the neurovascular hypothesis, is reduced in AD and leads to an increased accumulation of Aβ in the brain [75,101,123].

One of the main receptors responsible for uptake and subsequent clearance of Aβ through the BBB is the low density lipoprotein receptor-related protein 1 (LRP1). Endothelial specific knockout of LRP1 in mice was found to significantly reduce the clearance of Aβ through the BBB leading to increased soluble Aβ in the brain, and deficits in memory and spatial learning [123]. The LRP1 receptor is susceptible to ectodomain shedding, which results in the production of a transmembrane fragment and a soluble LRP1 protein. This soluble LRP1 maintains its ability to bind ligands but lacks the ability to internalise and

transcytose ligands through the cell [342]. This shedding process can be induced by exposure to Aβ although the precise mechanisms by which the shedding of LRP1 occurs as a result of exposure to Aβ remain unknown.

Our study identified an apoE dependent effect on the Aβ-induced shedding of LRP1 where the apoE2 and apoE3 isoforms were more effective than the apoE4 isoform at protecting the ectodomain shedding of LRP1. We also observed APOE allele dependent transport of Aβ through the BBB in mice, which was inversely correlated with the levels of soluble LRP1, suggesting that shedding of LRP1 may negatively impact the BBB clearance of Aβ. In addition, we identified APOE dependent expression of LRP1 in the brain and cerebrovasculature in human samples, with non-demented samples displaying increased expression when compared to AD samples. Interestingly, the ND APOE4 samples displayed the highest LRP1 expression, suggesting that this increase in expression may be compensating for the risk factor associated with the APOE4 genotype.

The clearance of Aβ through the BBB is a multistage process which is also dependent on the efficient trafficking of Aβ through the endosomal compartments after the initial internalisation. Therefore, we investigated whether the uptake of Aβ and endosomal trafficking of Aβ was altered as a result of the different apoE isoforms. We observed an increase in Aβ internalisation in cells exposed to apoE4 which, at first, appears to contradict the findings in Chapter 2 where APOE4 mice demonstrated reduced clearance through the BBB. However, the endosomal trafficking of Aβ in HBMECs also appears to be influenced by the apoE genotype present. It has previously been established that the endosomal transport of proteins, and the endosomes themselves, are dysfunctional in AD, with an increase of early endosome number and size [250,252]. It has also been suggested that dysfunction of the endosomal system could be one of the earliest detectable pathologies associated with AD and may therefore be one of the driving pathologies [250]. However, this defective trafficking in endosomes has also been identified in the brains of Down's Syndrome patients, a disease which is also associated with an increase

of Aβ in the brain [250,251,343]. This suggests that altered endosomal trafficking may occur as a result of the high levels of Aβ, indicating that dysfunctional protein trafficking may be a secondary pathology in both AD and Down's syndrome and not the primary cause. Regardless, our studies suggest that this dysfunction may be exacerbated by the apoE isoforms, with Aβ and apoE4 in combination causing an increase in the number and size of early endosomes in brain endothelial cultures. Interestingly, these enlarged early endosomes have previously been identified in AD brains and can be induced experimentally by disruption of downstream endosomal compartments, resulting in accumulation of Aβ in the early endosomes [250,254]. In contrast, cells exposed to the apoE2 isoform had fewer and smaller early endosomes. In comparison, we observed no apoE dependent effect on the recycling endosomes. Therefore, we conclude that while the apoE2 treated cells may endocytose Aβ at a slower rate than the apoE4 treated cells, the absence of swollen early endosomes, which are indicative of Aβ accumulation and dysfunctional trafficking mechanisms, suggests apoE2 must be more efficient at directing Aβ to the luminal surface of the cells, thus resulting in an increase in Aβ transported across the BBB.

Although the intracellular trafficking of Aβ in the cerebrovasculature appears to be apoE dependent, and modulation of these mechanisms could feasibly increase clearance of Aβ through the BBB, pharmacological targeting of these processes may prove challenging as a result of the ubiquitous nature of intracellular trafficking in all cell types. In addition, as previously discussed, the dysfunctional endosomal trafficking may only be a secondary pathology, which occurs as a result of a high Aβ burden. As such, the use of this therapeutic approach with the aim of slowing or even halting the progression of AD may be limited. In light of this, increasing the initial internalisation of Aβ at the BBB through increasing the expression of LRP1 may be a more attractive target to facilitate the clearance of Aβ through the BBB. While increasing the expression of LRP1 through stimulating its transcription would appear to be the most obvious approach, this avenue of intervention has previously proven challenging due to a narrow therapeutic window [344]. Therefore, we propose that inhibition of the sheddases responsible for the ectodomain shedding of LRP1 may be a more viable approach to maintain a

sufficient population of functional LRP1, thus enabling the clearance of Aβ across the BBB. We identified several prospective proteases which regulate the shedding of LRP1 in HBMECs, including the α-secretase ADAM10 and the matrix metalloproteinase MMP9, and assessed whether inhibition of these proteases was a viable therapeutic approach to facilitate the clearance of Aβ through the BBB. In addition, due to the large influence that the apoE isoforms have on risk of developing AD and the previously described clearance of Aβ through the BBB, we also assessed whether the activities of these sheddases were influenced by the different apoE isoforms.

ADAM10 is believed to be the main constitutive α-secretase which is responsible for the initial step in the non-amyloidogenic processing of the amyloid precursor protein (APP) in the brain [63,64]. However, it has previously been found to have enzymatic activity against LRP1 [146]. Initially we investigated whether inhibition of ADAM10 reduced Aβ induced shedding of LRP1 and could therefore be used to increase the increase the LRP1 dependent transport of Aβ across the BBB. We demonstrated that inhibition of ADAM10 reduced the shedding of LRP1 and increased the clearance of Aβ through the BBB in both *in vitro* and *in vivo* paradigms. However, a primary concern in the use of an ADAM10 inhibitor, particularly for the treatment of AD, is the effect it may have on the processing of APP as inhibition of the α-secretase non-amyloidogenic pathways could feasibly result in diversion of APP down the amyloidogenic pathway and the increased production of Aβ. Interestingly, treatment did not significantly impact the processing of APP down the non-amyloidogenic pathway, suggesting that either the α-secretase activity was compensated for by other members of the family, or that the inhibitor was not reaching the site of APP processing at high enough concentrations to have an impact. In addition, the newer generation of ADAM10/17 inhibitors have shown no signs of side effects that were previously associated with the inhibition of ADAM10 or ADAM17, suggesting that this may be a viable and relatively safe avenue of therapeutic intervention [266,345].

However, there are indications that the ADAM10 mediated shedding of LRP1 is apoE independent suggesting that it may not sheddase responsible for the apoE dependent levels of soluble LRP1. For example, cell-free activity assays demonstrated that apoE2 facilitated the cleavage of a fluorescent substrate to a greater degree when compared to the apoE3 and apoE4 isoforms. This activity profile is in contrast to the apoE mediated shedding of LRP1 outlined in Chapter 2, where the apoE2 isoform protected LRP1 from shedding. If ADAM10 was the primary driver of LRP1 shedding in the cerebrovasculature, we would witness an increase in the shedding of LRP1 as a result of apoE2. As this does not appear to be the case, we must conclude that while ADAM10 is capable of inducing the shedding of LRP1, it must not be the main driver of the apoE dependent mechanism for LRP1 clearance of Aβ through the BBB. However, while this ADAM10-apoE interaction may not be involved in this process, it may prove to be of particular interest with regard to the processing of APP by α-secretases down the non-amyloidogenic pathway. For instance, these data suggest that the apoE2 isoform increases the activity of ADAM10 which may result in increased processing of APP through the non-amyloidogenic pathway and less Aβ production when compared to the apoE4 isoform. Regardless, while it is clear that ADAM10 is capable of inducing LRP1 shedding, it did not appear to be the relevant sheddase responsible for in the apoE-dependent shedding of LRP1 at the cerebrovasculature. Therefore, we assessed the interaction between another sheddase, MMP9, and the apoE isoforms with regards to the shedding of LRP1.

Multiple pathologies in AD are associated with an increase in the expression of MMP9 [298,310,314]. As such, inhibition of MMP9 may be beneficial on many fronts, with existing studies demonstrating its ability to ameliorate Aβ-induced cognitive deficits and neurotoxicity [316,323], lower microglial activation [315], and protect the BBB from degradation [309,317,324]. However, little investigation has been done into MMP9 within the scope of apoE mediated pathology. To assess whether MMP9 is the pathological driver in the apoE dependent shedding of LRP1, we selectively inhibited MMP9 *in vitro*. This resulted in a significant reduction in the levels of soluble LRP1, suggesting that the modulation of MMP9

may be a viable target to increase the clearance of Aβ through the BBB by blocking LRP1 shedding. In contrast to the apoE-ADAM10 activity profile, MMP9 was inhibited by the apoE isoforms, albeit to varying degrees. In this paradigm, apoE2 demonstrated the highest levels of inhibition and apoE4 the lowest. This apoE isoform-dependent activity profile was in agreement with the pattern of apoE-mediated shedding of LRP1 outlined in Chapter 2, suggesting that MMP9 may be the pathological driver for the apoE-mediated shedding of LRP1 in the brain, thus, the cause of the reduced clearance of Aβ through the BBB witnessed in our study.

In support of this, inhibition of MMP9 increased the transport of Aβ through the BBB in an *in vitro* BBB model, leading us to advance these findings into an *in vivo* model. Previously, have demonstrated that APOE4 mice, following intracranial administration of exogenous Aβ, demonstrated significantly lower levels of Aβ in the plasma when compared to APOE3 mice. However, after treatment of APOE4 mice with an MMP9 inhibitor, APOE4 mice had comparable levels of Aβ in the plasma as untreated APOE3 mice, indicating an increase in the BBB clearance of Aβ. This suggests that blocking the shedding of LRP1 via MMP9 inhibition may be of particular use in individuals with the APOE4 allele who have increased risk of developing AD. As previously mentioned, treatments that show efficacy in individuals with the APOE4 allele are much sought after, with a number of otherwise promising therapeutic approaches showing either no efficacy or increased side effects in this genotype [60,62,346–348]. However, it should be noted that the inverse has also been found, with APOE4 carriers showing either a greater response to treatment than the other APOE carriers or no significant difference [349–351]. Regardless, inhibition of MMP9 would likely be beneficial in all APOE genotypes as a result of the multiple AD pathologies in which MMP9 is implicated, including the shedding of LRP1 at the BBB as outlined in this thesis.

We also identified an increased expression of MMP9 in cerebrovascular cells as a result of Aβ exposure. This is of particular interest, as accumulation of Aβ in AD may result in a self-perpetuating increase in MMP9 expression and release, leading to more LRP1 shedding and therefore less clearance of Aβ

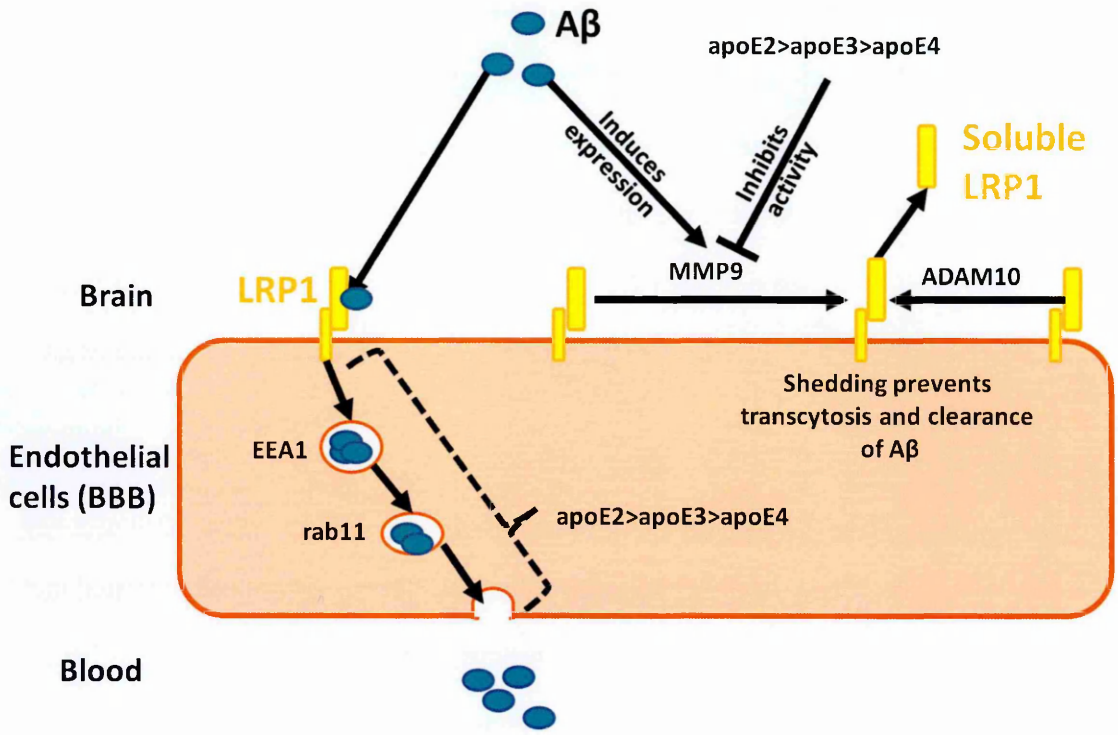

Figure 6.1 Proposed mechanism for the involvement of apoE in the regulation of Aβ clearance through the BBB. The shedding of LRP1 at the cell surface results in the formation of a soluble LRP1 fragment which is no longer able to transcytose Aβ. Two of the proteases responsible for this shedding are ADAM10 and MMP9, the latter of which was found to be apoE dependent (apoE2<apoE3<apoE4) and had expression which was inducible by Aβ. Once Aβ binds to LRP1, it is internalised and transported through the early endosomal compartments (EEA1) to the recycling compartments (rab11) where it is subsequently released into the plasma. Clearance of Aβ through the BBB occurs in an apoE isoform specific manner (apoE2>apoE3>apoE4). The presence of apoE3 or apoE4 results in an accumulation of Aβ in early endosomes resulting in an increase in the number and a swollen morphology of these endosomes.

through the BBB. This circular process could feasibly exacerbate the MMP9 mediated pathology at the BBB, leading to increased brain Aβ accumulation and MMP9 mediated pathology. The ability to break

this cycle through the inhibition of MMP9 could have an enormous impact on the progression of AD. However, the use of MMP9 inhibitors as a therapeutic strategy has previously been hampered by the high incidence of side effects of broad spectrum MMP inhibitors such as musculoskeletal pain and inflammation [326–328]. Fortunately, more recent studies that selectively targeted MMP9 appear to circumvent the side effects associated with broad spectrum MMP inhibition while retaining the efficacy, suggesting that selective inhibition of MMP9 remains a viable therapeutic strategy [329,330].

6.3 Conclusions

Despite the increasing prevalence of AD and a growing elderly population, there is a lack of disease modifying therapies with all currently approved therapies being directed towards palliative care. In addition, the current therapies have only shown limited efficacy and are often only marginally effective in a subset of AD patients [13–15]. While the magnitude and scale of the problem has now been recognised, with the World Health Organisation declaring the treatment of dementias a public health priority in 2012, there remains an urgent need to diversify the therapeutic avenues being investigated for the treatment of AD. This study focuses on a novel approach to facilitate the clearance of Aβ from the brain through the BBB. In addition, we also investigated whether the well associated risk factor for AD, the APOE4 allele, has any mechanistic involvement in the LRP1 mediated clearance of Aβ at the BBB.

We identified several mechanisms that were influenced by the apoE isoform, such as altered endosomal trafficking of Aβ in the early endosomes as well as an apoE dependent mechanism that resulted in the ectodomain shedding of LRP1(Figure 6.1). We believe that inhibition of LRP1 shedding presents itself as an attractive and effective means of increasing the clearance of Aβ across the BBB by increasing the functional the full length LRP1 population. Of the proteases investigated in this study, MMP9 proved to be the most promising target for therapeutic intervention, primarily as a result of its strong association with several AD pathologies. In addition, MMP9 activity was found to be mediated in an apoE isoform

dependent manner. This novel association could prove particularly beneficial to individuals with the APOE4 allele who are most at risk of developing AD.

To our knowledge, this is the first demonstration that the proteases responsible for the ectodomain shedding of LRP1 can be successfully modulated to facilitate the clearance of Aβ through the BBB. This approach represents a novel therapeutic avenue for the treatment of AD that may be invaluable for reducing the accumulation of Aβ in the brain. In addition, there has never been a therapeutic approach that is specifically targeted toward restoring the BBB clearance of Aβ. This strategy may open up a new category of viable therapeutics that specifically targets dysfunctional BBB clearance. We believe that following this line of enquiry may be of the upmost importance if a successful therapy for AD is to be developed.

6.4 Future directions

The findings from this thesis support the targeting of the proteases involved in the shedding of LRP1. In particular, the inhibition of MMP9 may prove to be a particularly desirable therapeutic avenue as a result of the multiple AD pathologies in which it is involved. To our knowledge, there have currently been no studies investigating the impact of MMP9 inhibition in an AD mouse model. Therefore, future studies will investigate whether treatment with the MMP9 selective inhibitor SB-3CT is able to alter the AD phenotype in a mouse model of AD by increasing the BBB clearance of Aβ. In addition, assessment of MMP9 expression in the vasculature of non-demented and AD individuals stratified by APOE genotype may prove to be particularly interesting as it may reveal APOE dependent expression patterns. Findings from these studies may give additional rationale for the increased risk of developing AD that the APOE4 allele confers.

Chapter 7 Acknowledgments

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Chapter 9 : Appendix

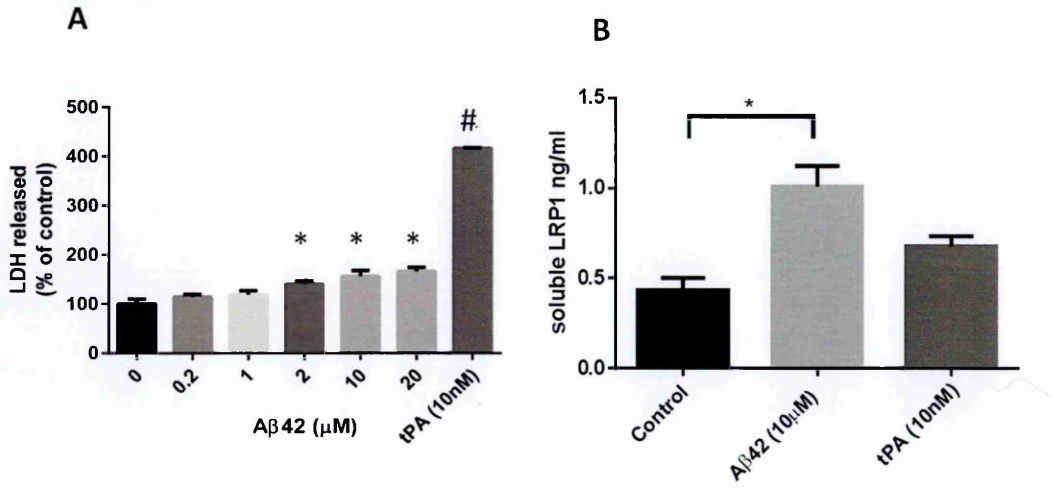

Figure 9.1 (A) Release of Lactate Dehydrogenase by HBMECs after Aβ42 treatment. HBMECs were incubated with Aβ42 for 48hours before the media was collected and tested for the presence of the well known cytoxic marker, LDH (Roche Diagnostics, Germany) according to the manufacturer's instructions. At concentrations higher than 1μM Aβ42, a significant increase in the levels of LDH was detected. The largest increase was witnessed in the tPA positive control (10nM) which had significantly higher levels of LDH compared to all other treatments. No significant difference was detected between control and Aβ42 at any concentration. (B) Shedding of LRP1 in HBMECs after treatment with Aβ42 (10μM) and tPA (10nM). Treatment with Aβ42 significantly increased the levels of soluble LRP1 with exposure of HBMECs to tPA resulting in a small increase that did not reach statistical significance. Values represent mean ± SEM (n = 3) and are expressed as % change LDH from control. Statistical significance by assessed by one-way ANOVA followed by Tukey's *post hoc* analysis. *$p<0.05$ compared to 0μM Aβ42, #$p<0.0001$ compared to all other treatments.

Group	ID	Sex	Age	APOE	PMI (hours)	Clinical Summary	Plaque total	Braak Score	Cerad NP	Source
AD	001	F	76	4/4	3	AD	13.75	IV	pAD	BSHR
AD	002	F	96	3/3	2.75	AD	12.5	V	pAD	BSHR
AD	03	F	77	4/4	4	AD	13.25	V	definite AD	BSHR
AD	004	M	86	3/3	3	AD, PD	14.25	V	pAD	BSHR
AD	005	F	87	3/3	3	AD	13.5	V	definite AD	BSHR
AD	006	F	84	3/3	1.83	pAD	14.5	VI	definite AD	BSHR
AD	007	M	86	3/3	2.16	pAD	14.5	IV	definite AD	BSHR
AD	008	M	76	4/4	4	AD or PiD	11.75	V	definite AD	BSHR
AD	009	F	85	3/3	1.5	pAD	14	VI	definite AD	BSHR
Control	010	F	83	2/2	4.83	Control, D, action T	10	II	possible AD	BSHR
AD	011	F	96	3/3	3	Possible AD	13.75	IV	pAD	BSHR
Control	012	M	91	3/3	1.5	Control	0	II	not AD	BSHR
AD	013	F	86	3/3	2.5	AD	15	V	definite AD	BSHR
Control	014	M	82	3/3	3	Control, mPD, S	4	III	not AD	BSHR
AD	015	F	75	3/3	2.83	CD	15	V	definite AD	BSHR
Control	016	M	85	3/3	3.5	Control, pMCI	0	I	not AD	BSHR
Control	017	M	92	3/3	3	ND control, T	8	III	possible AD	BSHR
AD	018	F	92	4/4	2.83	AD	14.5	VI	definite AD	BSHR
Control	019	M	89	3/3	5.5	Normal control	0	II	not AD	BSHR
AD	020	F	82	3/3	4	AD	15	VI	definite AD	BSHR
AD	021	M	72	4/4	3	AD	15	V	definite AD	BSHR
Control	022	M	81	3/3	2.25	ND control, T	0	III	not AD	BSHR

Control	023	M	80	3/3	2.5	ND control	0	III	not AD	BSHR
Control	024	M	38	3/3	3	ND control	0	0	not AD	BSHR
Control	025	M	80	2/2	3.5	ND control, PC	4.5	III	not AD	BSHR
AD	026	F	88	4/4	2.28	AD, PD, NOS	13	VI	definite AD	BSHR
Control	027	M	38	2/2	9	Control	NA	NA	NA	M
AD	028	M	83	4/4	3	AD	10	V	definite AD	BSHR
Control	029	F	91	2/2	4	ND control	11	IV	possible AD	BSHR
Control	030	M	49	4/4	10	ND control	NA	NA	NA	M
Control	031	F	33	3/3	20	ND control	NA	NA	NA	M
Control	032	M	32	4/4	13	ND control	NA	NA	NA	M
Control	033	F	34	4/4	12	ND control	NA	NA	NA	M
Control	034	F	60	4/4	18	ND control	NA	NA	NA	M
Control	035	M	58	4/4	17	ND control	NA	NA	NA	M
Control	036	M	49	4/4	5	ND control	NA	NA	NA	M
Control	037	M	51	4/4	25	ND control	NA	NA	NA	M
Control	038	F	54	2/2	11	ND control	NA	NA	NA	M
Control	039	F	102	2/2	1.67	ND control	0	II	not AD	BSHR
Control	040	M	78	2/2	1.66	ND control	9.75	I	not AD	BSHR
AD	041	M	68	4/4	2	AD	15	VI	definite AD	BSHR
Control	042	F	81	4/4	3	Control	7.5	II	not AD	BSHR
AD	043	F	93	4/4	2.5	pAD	14	VI	definite AD	BSHR
Control	044	F	70	4/4	2	Control	8.25	I	not AD	BSHR
AD	045	M	83	4/4	2.83	AD	15	VI	definite AD	BSHR
Control	046	F	76	3/3	2.75	Control	4.6	I	possible AD	BSHR

Control	047	M	84	3/3	2.5	ND Control	9.25	III	possible AD	BSHR
Control	048	F	91	2/2	2.5	Control; pMCI	1	III	not AD	BSHR
AD	049	F	73	2/2	7.3	AD	6.1	NA	NA	MtS
AD	050	F	107	2/2	15.1	AD	4.96	NA	NA	MtS
Control	051	F	80	2/2	3.8	ND Control	0	NA	NA	MtS
Control	052	F	94	2/2	2.1	ND Control	0	NA	NA	MtS

Table 9.1 Demographics for human inferior frontal gyrus samples. MI=post mortem interval, ND= Non-demented, PAD=Alzheimer's disease, pAD=probable AD, PD=Parkinson's disease, mPD=mild Parkinson's disease, PiD=Pick's disease, MCI=mild cognitive impairment, pMCI= possible mild cognitive impairment, T=tremor, S=Stroke, PC=pancreatic cancer, CD=corticobasal degeneration, BSHR=Banner Sun Health Research Institute, M=University of Maryland, MtS=Mount Sinai Hospital, NA=not available